

EFFECTS OF COX-2 INHIBITORS AND TRANSLOCATOR PROTEIN DRUG LIGANDS ON PROGESTERONE PRODUCTION BY MOUSE LEYDIG TUMOR CELLS

By

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Abstract

Cyclooxygenase 2 (COX-2) inhibitors and translocator protein 18 kDa (TSPO) drug ligands have been shown to have stimulatory effects on steroid production. Given that their mechanisms of action differ, we hypothesized that a combination treatment of both TSPO drug ligands and COX-2 inhibitors might have additive or synergistic effects on steroid production in the Leydig cell. We tested the effects of each of four COX-2 inhibitors (Apigenin, DFU, NS398, Indomethacin) and two TSPO drug ligands (Ro5, FGIN) on steroid hormone (progesterone) production by MA-10 mouse Leydig tumor cells. The four COX-2 inhibitors had similar effects across all concentrations when inhibitor-treated cells were then incubated with luteinizing hormone (LH). When cells were treated with the COX-2 inhibitor and then incubated with dibutyl cAMP (dbcAMP), dose-response effects were seen. DFU and Indo were the two COX-2 inhibitors that had maximal effects on mean progesterone production. Treatment of cells with the TSPO drug ligands, Ro5 and FGIN, also resulted in increased progesterone production. However, we found no additive or synergistic effects of TSPO ligands and COX-2 inhibitors when cells were treated in the presence of LH or dbcAMP. Thus, although the mechanisms by which COX-2 inhibitors and TSPO drug ligands stimulate steroid formation differ, treatment of cells by combining the two classes of compounds had no greater effect on progesterone production than either alone. As yet, however, the possibility that these compounds could have additive or synergistic effects has not been assessed using primary cells or in vivo.

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Table of Contents

Abstract-----	Page ii
Acknowledgements-----	Page iii
Literature Review-----	Pages 1-32
Manuscript Introduction-----	Pages 33-37
Materials and Methods-----	Pages 38-40
Results-----	Pages 41-46
Discussion-----	Pages 47-51
Figures-----	Pages 52-67
References-----	Pages 68-73
Curriculum Vitae-----	Pages 74-77

LITERATURE REVIEW

Introduction

Steroid hormones play important roles throughout the life course. Alterations in the steroidogenic process can have dramatic effects on the human body regardless of at what age alterations occur. With aging, alterations in steroid production can cause problems in mental and physical functioning. In men, the decline in testosterone production that is characteristic of aging can cause symptoms that arguably decrease quality of life. Much work has been done examining how the steroidogenic machinery works to produce steroid hormones such as estrogen and testosterone. By better understanding how different cellular components work together to produce hormones, there is the potential for novel drug therapies to treat medical conditions arising from deficiencies in steroid hormone levels. One possible way to elevate testosterone in aging males is to provide testosterone exogenously. A more novel approach is to enhance testosterone production by the Leydig cells themselves with pharmacological intervention. Two potential classes of chemical compounds, translocator protein 18 kDa (TSPO) drug ligands and cyclooxygenase 2 (COX-2) enzyme inhibitors have recently been examined for this purpose. This review will outline the mechanisms behind steroidogenesis in the Leydig cells, as well as the therapeutic potential of TSPO drug ligands and COX-2 inhibitors. It is possible that when these two pharmacological agents are given to Leydig cells as a drug cocktail, they can have an additive or synergistic effect on steroidogenesis. If this is the case, then COX-2 inhibitors and TSPO drug ligands might offer a new avenue for pharmacological treatment for low steroid levels.

Steroidogenesis

Leydig Cells

In the testis, testosterone is produced by Leydig cells. In order to study steroid production experimentally, the appropriate models must be used. Various models for Leydig cell steroid production exist. Two that have been used extensively are MA-10 mouse Leydig tumor cells and primary cells, particularly from Brown Norway rats. Most of the experimental data in this thesis was derived using MA-10 Leydig tumor cells, but much of the background material that served as the basis for these experimental designs also came from rat models. Leydig cell changes throughout the lifetime have been well studied in rats, a common animal model used to study testosterone synthesis. Brown Norway rats in particular are an excellent animal model for the study of steroidogenesis due to their similarities in reproductive changes to humans, as well as the absence of tumors with aging and overall good general health (Chen et al., 2009). In rats Leydig cells can be classified as fetal Leydig cells that develop in utero, or as adult Leydig cells that develop during puberty. Fetal Leydig cells do not require luteinizing hormone (LH) for their development, although they do express the LH receptor and respond to LH stimulation. Adult Leydig cells are responsible for producing the testosterone needed to initiate and then maintain spermatogenesis (Chen et al., 2009). These cells form from a distinct four stage model of development: stem Leydig cells, progenitor Leydig cells, immature Leydig cells, and adult Leydig cells (Chen et al., 2009). These four different stages have characteristic cellular properties that distinguish them from the others. For example, Leydig cell specific markers such as the LH receptor and 3 β -hydroxysteroid dehydrogenase are not present in stem Leydig cells (Ge et al., 2006). Development of

Leydig cell markers such as the LH receptor and the P450 side-chain cleavage enzyme are hallmarks of the transition from stem Leydig cells to progenitor Leydig cells. The progenitor Leydig cells are able to produce steroids, and secrete mostly androsterone (Chen et al., 2009). Advancement to the immature Leydig cell stage is characterized by an expansion of the smooth endoplasmic reticulum and increased capacity for steroidogenesis. The primary steroid product is 5α -androstane- 3α , 17β -diol (ADIOL) (Chen et al., 2009). Adult Leydig cell development is noted when the cells begin to produce more testosterone than ADIOL, and these cells do not normally proliferate but can regenerate (Chen et al., 2009).

The ability of the Leydig cell to react to LH stimulation is a main component of the steroidogenic pathway. However, Leydig cell activity can be influenced by a variety of factors; LH signaling is not the only process that can have an effect on steroidogenesis. One of these factors is neuropeptide Y. It was found that injection of neuropeptide Y directly into the testes of Sprague-Dawley or Wistar rats blunted the testosterone response to human chorionic gonadotropin (hCG), suggesting that this peptide may act independently of LH (Allen et al., 2011). Possible mechanisms suggested for the decrease in testosterone include reduced delivery of hCG to Leydig cells or interference with testosterone release in the general circulation. The results of this experiment highlight the need for further investigation into neuropeptide Y as a potential influence on testicular activity through a local site of action (Allen et al., 2011). So while LH signaling is the main focus of the experiments detailed in this thesis, it is noted that various other mechanisms may also be utilized to enhance or effect steroid production in Leydig cells.

Testosterone Synthesis in the Male Reproductive System

Testosterone is produced in the male reproductive system by Leydig cells located in the testicular interstitium. As reviewed by Midzak et al. (2009), production of testosterone is in response to stimulation by the pituitary gonadotropin luteinizing hormone (LH). In adult Leydig cells, pulsatile secretion of LH by the pituitary gland into the peripheral circulation is the driving force behind testosterone production. Steroid synthesis in the Leydig cell is first activated by the binding of LH to the LH receptor on the cell membrane, with subsequent initiation of G-protein coupling to the LH receptor, activation of adenylate cyclase, increased intracellular adenosine 3',5'-cyclic monophosphate (cAMP) formation, and phosphorylation processes through protein kinase A. A simplified figure of the steroidogenic pathway is presented in **Figure 1** below. LH action has both short-term and long-term effects, in the latter case maintaining the expression of the steroidogenic enzymes (Midzak et al., 2009).

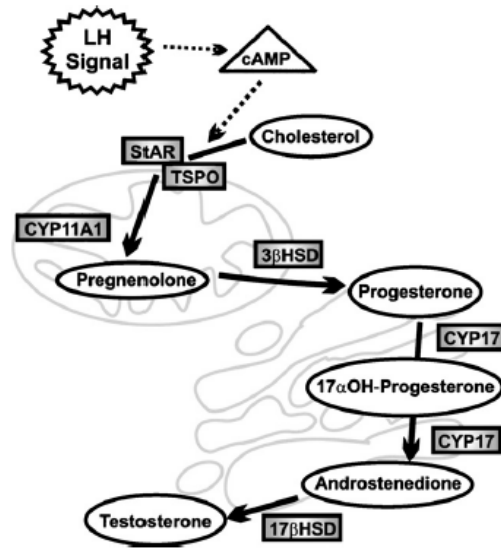


Figure 1: Diagram depicting the steroidogenic pathway. Signaling pathways that start the steroidogenic process are initiated when the Leydig cell responds to LH. Taken from *Leydig Cell Aging and the Mechanisms of Reduced Testosterone Synthesis* (Midzak et al., 2009).

The steroidogenic pathway in the rat has been well studied, with a distinct set of steps necessary for the production of testosterone. This process involves a signaling cascade that must be completed stepwise before testosterone can be produced. All steroid hormones are synthesized from cholesterol. The source of this cholesterol is either *de novo* synthesis from acetate in the endoplasmic reticulum, or the import of cholesterol that is bound to circulating lipoproteins (Papadopoulos and Miller, 2012). In humans, cholesterol can come from high density lipoproteins, or from low density lipoproteins derived from dietary sources. The latter is the main source of steroidogenic cholesterol. However, rodents differ from humans in that high density lipoproteins are the principal source of cholesterol for steroidogenesis (Papadopoulos and Miller, 2012). Low density lipoproteins are stored in droplets as cholesteryl esters that can fuse with lysosomes and be degraded to free the cholesteryl esters. The cholesteryl esters are then hydrolyzed to free cholesterol by lysosomal acid lipase, which

can then enter the steroidogenic pathway or become re-esterified for storage (Papadopoulos and Miller, 2012).

In order for cholesterol to be converted to another steroid, it must be shuttled to the inner mitochondrial membrane where the cholesterol side chain cleavage enzyme cytochrome P450 side chain cleavage (P450_{scc}) or cytochrome P450 11A1 (CYP11A1) is located. It is thought that TSPO is part of a complex of proteins residing in both the inner and outer mitochondrial membranes involved in this cholesterol transport (Rupprecht et al., 2010). This complex is referred to as the transduceosome, and includes proteins such as StAR and the voltage-dependent anion channel (VDAC-1) (Issop et al., 2013). Other components of the transduceosome include TSPO-associated protein 7 (PAP7), ACBD3 (acyl-CoA-binding-domain 3) and protein kinase A regulatory subunit 1 α (PKAR1A) (Papadopoulos and Miller, 2012). **Figure 2** shows the various proteins involved in the steroidogenic process, and how the components of the transduceosome interact with one another.

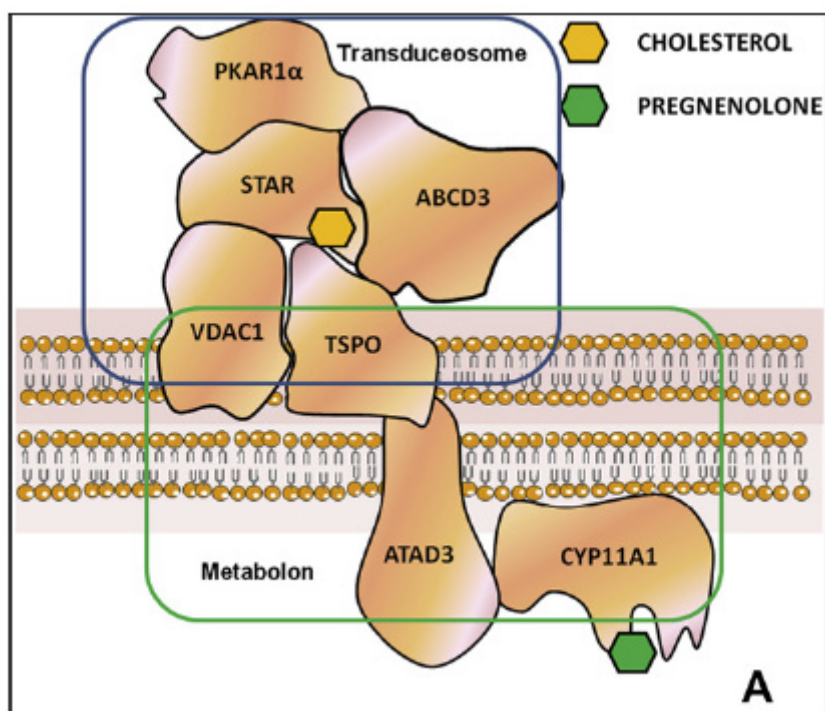


Figure 2: Various components of the transduceosome involved in steroidogenesis are shown. The transduceosome is named as such because it transduces the cAMP signal at the outer mitochondrial membrane. Taken from *Organelle Plasticity and Interactions in Cholesterol Transport and Steroid Biosynthesis* (Issop et al, 2013).

Cholesterol does not freely diffuse across the mitochondrial outer membrane through intermembrane space to the inner membrane (Midzak et al., 2011). The conversion of cholesterol to pregnenolone on the inner mitochondrial membrane by P450_{scc}/CYP11A1 is the primary point of post LH-receptor control during testosterone synthesis due to LH stimulation (Midzak et al., 2009). The transport of cholesterol from cellular stores to the inner mitochondrial membrane is cAMP dependent (Midzak et al., 2009). In response to stimulation by LH, cAMP is produced and stimulates the transfer of cholesterol from outside

the mitochondria into the inner mitochondrial membrane where the P450 cholesterol side-chain cleavage enzyme (P450_{scc}/CYP11A1) metabolizes it to pregnenolone (Midzak et al., 2009). Conversion of cholesterol to testosterone in the rat Leydig cell involves two principal classes of enzymes: the cytochrome P450 proteins (P450_{scc}/CYP11A1 and CYP17) and the hydroxysteroid dehydrogenases (3 β HSD and 17 β HSD). In a stepwise pattern first pregnenolone is synthesized from cholesterol, followed by progesterone, then 17 α -hydroxyprogesterone/androstenedione, followed lastly by testosterone (Midzak et al., 2009). Pregnenolone moves out of the mitochondria and to the smooth endoplasmic reticulum where it is then metabolized to progesterone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD). Then, 17 α -hydroxylase/ C17-20 lyase (CYP17) metabolizes progesterone to androstenedione. In the last step, androstenedione is metabolized to testosterone by type 3 17 β -hydroxysteroid dehydrogenase (17 β -HSD3) (Midzak et al., 2009).

The LH receptor is a glycoprotein member of the G protein-coupled receptor family (Midzak et al., 2009). When LH binds to the LH receptor, stimulatory G proteins (G_s) are activated, subsequently relaying a signal to release of GDP by the α subunit of the G protein (Midzak et al., 2009). When the α subunit becomes active (binds GTP), it translocates and activates the effector molecule adenylyl cyclase to generate cAMP (Midzak et al., 2009). Cholesterol transport across the mitochondrial membrane is dependent on cAMP stimulation and involves TSPO and the steroidogenic acute regulatory protein (StAR) (Midzak et al., 2009). Cholesterol is transferred to the inner mitochondrial membrane in order to be cleaved by P450_{scc}/ CYP11A1 to pregnenolone (Zirkin and Tenover, 2012).

cAMP and Leydig Cells

It is known that the amount of cAMP produced in cells due to LH stimulation is reduced in aged compared to young cells (Chen et al., 2004). It is thought that reduced cAMP could be a potential player in the demonstrated age related reduction in steroidogenesis. Indeed, it was found that cells from aged rats cultured with dbcAMP were able to produce testosterone at levels similar to those seen in young cells, and had increases in StAR and P450_{scc}. Old cells that were treated with dbcAMP for three days produced double the amount of testosterone of cells cultured with LH (Chen et al., 2007).

LH Signaling and Cyclooxygenases

Prostaglandins play a critical role in a variety of functions throughout the body. Prostaglandins are important in the body because of their role in mediating pain and because they are implicated in a variety of conditions such as hypertension, cancer, inflammation, and normal function of the female reproductive system (Fortier et al., 2008). Prostaglandins act locally in a paracrine or autocrine fashion, and have been found to be produced by all nucleated cells of the body (Fortier et al., 2008). Cyclooxygenases catalyze the conversion of arachidonic acid to PGG₂ and H₂, which is an initial step in prostaglandin biosynthesis (Fujimoto et al., 2004). Two isoforms of cyclooxygenase have been well studied, respectively named COX-1 and COX-2. Whereas COX-1 is a ubiquitous enzyme that appears to be involved in a variety of homeostatic functions such as in the kidney, COX-2 is thought to be synthesized usually in response to inflammatory stimuli (Fujimoto et al., 2004; Seta et al, 2009). COX-2 is considered the inducible isoform of cyclooxygenase, and is known to be regulated by factors such as cytokines or tumor promoters (Sirois and Richards,

1992; Fortier et al., 2008). While similar, COX-1 and COX-2 also appear to be involved in different cellular activities, specifically in relation to the types of prostaglandins they produce (Seta et al., 2009). COX-2 has also been shown to produce prostaglandins from relatively low levels of arachidonic acid compared to COX-1 (Parent et al., 2003). Clinically, cyclooxygenases play an important role in pharmacological intervention for a variety of diseases. Nonsteroidal anti-inflammatory drugs (NSAIDs) are known for their anti-inflammatory, analgesic, and antipyretic properties, which can be attributed to inhibition of COX-2 derived prostaglandin production at the site of inflammation (Seta et al., 2009). NSAIDs have been used to pharmacologically target prostaglandin production in the body for more than a century (Fortier et al., 2008). It is also known that the production of reactive oxygen species and reactive nitrogen species is characteristic of the inflammatory response, which can affect cellular function (Fujimoto et al., 2004). Experiments examining the effects of reactive oxygen and nitrogen species on COX-1 and COX-2 activities found that these species can have different modulatory effects on these enzymes, with the two isoforms showing different sensitivity to the presence of reactive oxygen and nitrogen species. This is important because it is known that a significant amount of reactive oxygen species and nitrogen species are formed in inflammatory cells that also contain high amounts of COX-2 (Fujimoto et al., 2004). This relationship is significant as COX-2 and its inhibitors have also been known to affect steroidogenesis in Leydig cells.

It is known that LH acts principally through a cAMP dependent pathway to stimulate cholesterol transport and steroidogenic enzymes (Chen et al., 2007). In addition to cAMP synthesis and signaling, however, LH also induces the release of intracellular arachidonic acid (Midzak et al., 2009). It is thought that arachidonic acid is released from cell

membrane lipids through the action of phospholipase A2 and/or acyl-CoA synthetase (Chen et al., 2007). Arachidonic acid is then metabolized by lipoxygenase, epoxygenase, or cyclooxygenase stores in the cell (Midzak et al., 2009). It is thought that expression of cyclooxygenase 2 (COX-2) in the Leydig cells plays a role in the declining levels of testosterone production seen in aging Leydig cells (Chen et al., 2007). COX-2 may in fact suppress steroidogenesis since inhibition of COX-2 was shown to increase StAR and progesterone production in dbcAMP stimulated MA-10 Leydig tumor cells (Wang et al., 2003).

When examining the relationship between COX-2 and LH, it was found that two hours of treatment with LH increased COX-2 activity up to 240% of the basal level in primary rat Leydig cells. This effect was also seen when cells were incubated with dbcAMP, along with an increase in testosterone production. When treated with a PKA inhibitor the stimulatory effects were inhibited, suggesting that cAMP-PKA signaling plays a role in LH stimulation of COX-2 similarly to how it plays a role in testosterone production stimulation. In addition, there was a significant increase in COX-2 protein content that was age-dependent but also independent of testis weight and testicular germ cell count (Chen et al., 2007).

Cyclooxygenase-2 Inhibitors

A variety of COX-2 inhibitors have been used to study how COX-2 activity influences steroidogenesis. One way to do this is to use COX-2 inhibitors to determine how COX-2 activity affects LH or cAMP- stimulated steroidogenesis. When the COX-2 inhibitor indomethacin was used to treat MA-10 mouse Leydig cells in conjunction with dbcAMP and therefore inhibit steroidogenesis, increased StAR protein levels and coinciding progesterone

increase was seen in a concentration- dependent manner. However, this effect was not seen when MA-10 cells were treated with indomethacin alone (Wang et al., 2003). Using indomethacin to inhibit COX activity with dbcAMP also resulted in a significant increase in StAR mRNA levels and StAR promoter activity, suggesting that dbcAMP is critical for the stimulatory effects of indomethacin. Similar effects were seen when the COX-2 inhibitor NS398 was used. Within the NS398 treated MA-10 cells, it was found that dbcAMP treatment also significantly increased PKA activity and StAR protein expression. Together, the results from this study suggest that COX activity inhibition increases the sensitivity of steroidogenesis to cAMP stimulation in MA-10 Leydig cells, and lowers the concentration of cAMP necessary for maximal steroid production (Wang et al., 2003).

Clinically, COX-2 inhibitors have been explored as a therapeutic alternative to nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs are associated with major side effects on renal function and gastrointestinal events. NSAIDs are a heterogeneous class of drugs with a wide range of selectivity toward COX-1 and COX-2 (Seta et al., 2009). The theory behind COX-2 inhibitor use is that sparing of inhibition of COX-1 activity could result in increased safety than when both COX-1 and COX-2 are inhibited (Schnitzer, 2001). Selective COX-2 inhibitors were developed on the principle that COX-1 is the constitutively expressed isoform of the cyclooxygenase enzyme responsible for housekeeping functions such as gastrointestinal cytoprotection, platelet aggregation, parturition, and renal homeostasis, whereas COX-2 is expressed only in response to inflammatory and mitogenic stimuli (Seta et al., 2009). Data from clinical trials has shown that COX-2 inhibitor use has shown increased gastrointestinal safety, but their use still coincides with some of the same side effects seen with dual COX inhibitors (NSAIDs) (Schnitzer, 2001). Chronic use of

COX-2 inhibitors has also been associated with a mild to moderate increase in blood pressure as well as papillary necrosis and renal failure (Seta et al., 2009). COX-2 inhibitors also may have a possible association with an increased risk of cardiovascular and cerebrovascular disease in clinical trials, which eventually led to their withdrawal from the market (Hennekens et al., 2014; Seta et al., 2009). While COX-2 inhibitor use comes with serious side effects, this approach nevertheless remains to be explored further as a potential mediator in the steroidogenic response.

Steroidogenesis in Experimental Models

When studying steroidogenesis in various models, it should be noted that different animal and cell models have characteristics that make one or the other better suited for specific studies. For example, two of the most common models for steroidogenesis, MA-10 tumor Leydig cells and primary Leydig cells from Brown Norway rats, produce different steroids as their final product. Primary cells produce testosterone, whereas MA-10 cells produce progesterone as a result of decreased CYP17 expression. This makes MA-10 cells an inappropriate model for studying the later steps required for testosterone production (Midzak et al., 2011). However, past experiments have shown that mitochondrial ATP synthesis is critical for steroidogenesis in both models but the two still have marked differences. For example, glycolysis is a significant source of cellular ATP in MA-10 cells while primary cells depend almost completely on mitochondrial respiration for their energy sources. It was also found that ATP levels in MA-10 cells showed greater sensitivity to glycolytic inhibition compared to ATP levels in primary cells. This same experiment also showed that MA-10 cells utilize a greater proportion of glycolytic ATP than primary cells for cholesterol transport, but mitochondria-derived ATP still remains critical in these cells.

Together these results highlight why data derived from experiments with MA-10 cells cannot necessarily be applied to primary cells (Midzak et al., 2011). This is an important fact to remember when translating work done from one model to another.

MA-10 Cells and Oxidative Stress

In order to examine the relationship between the redox environment and the susceptibility of MA-10 cells to oxidative stress, an altered redox environment was created by exposing the cells to buthionine sulfoximine (BSO) or diethyl maleate (DEM) in order to decrease glutathione (GSH), after which cells were exposed to the prooxidant tert-butyl hydroperoxide (t-BuOOH) (Chen et al., 2010). Cells with depleted GSH (an antioxidant molecule that plays a role in maintenance of the thiol redox balance in cells) were exposed to t-BuOOH. Reactive oxygen species concentration was significantly increased intracellularly, and this change also resulted in a significant reduction in progesterone production (Chen et al., 2010). However, inhibition of p38 (a MAPK family member) phosphorylation prevented the t-BuOOH driven reduction in progesterone production in GSH depleted cells (Chen et al., 2010). This experiment showed that MA-10 cells with a reduced GSH pool were more sensitive to acute oxidative stress compared to cells with normal or increased GSH levels, and that this susceptibility to oxidative stress is mediated by redox-sensitive MAPK signaling pathways. Because p38 MAPK signaling is an important regulator of COX-2 expression, the results of this experiment suggest a potential regulatory network involving oxidative stress, p38 MAPK, COX-2, and steroidogenesis (Chen et al., 2010).

Steroidogenesis and Aging

Testosterone and Aging

In humans and in Brown Norway rats, there are reduced serum testosterone concentrations seen with aging (Chen et al., 2009). Alterations in testosterone production are not just due to action in the testes, but can also be attributed to changes in the hypothalamic-pituitary axis (Veldhuis et al., 1997; Zirkin and Tenover, 2012). . In rats, it has been shown that the loss of Leydig cells does not explain age-related reduction in serum testosterone levels (Chen et al., 1994). Rather, there are changes in Leydig cell function, as in vitro studies have shown that Leydig cells isolated from aged rat testes produce less testosterone than their young counterparts in response to LH (Chen et al., 1994).

Declining testosterone production with age could be due to a reduction in steroidogenic enzyme activities, the amount of substrate available for steroidogenesis, or a combination of these two factors (Culty et al., 2002). When Leydig cells from young and old rats were incubated with human chorionic gonadotropin (hCG), dbcAMP, or 22-hydroxycholesterol, old cells always produced less testosterone than young cells, but cells treated with 22-hydroxycholesterol produced testosterone at a level 5-10 times greater than hCG treated cells. This suggests that the availability of substrate may be a limiting factor in steroidogenesis (Culty et al., 2002). In order to indirectly measure the hormonally induced cholesterol pool, young and old Leydig cells were treated with hCG and aminoglutethimide (AMG), a P450_{scc} inhibitor, and then mitochondria were isolated in the presence of AMG. Upon removal of AMG, the mitochondria of old cells produced only 80% of the steroid of mitochondria from young cells. This suggested that old Leydig cells are able to recruit less

cholesterol into the mitochondria than young cells (Culty et al., 2002). It was also found that TSPO mRNA and protein expression were decreased in old cells compared to young ones, suggesting that alterations in cholesterol transport may be a critical factor in declining testosterone production with age in Brown Norway rat Leydig cells. Altogether, these results suggest that declining cholesterol transport into the mitochondria and changes in TSPO could be factors in the declining testosterone production characteristic of aging.

Experimental evidence has shown that the reduced testosterone levels of aged males are not due to reduced LH levels (Zirkin and Tenover, 2012). This is supported by experimental evidence in Brown Norway rats showing that serum LH levels do not significantly change with age (Chen et al., 1994). In fact, the main factor in the decline of serum testosterone in aged males is the decreased ability of the testes to synthesize testosterone in response to LH (Zirkin and Tenover, 2012). Specifically, the testes of aged Brown Norway rats produced significantly less testosterone compared to the testes of young rats when both were perfused in vitro with maximally stimulating LH. This suggested that the reduced serum testosterone seen in aged rats was due to reduced testosterone production by the Leydig cells (Zirkin, et al., 1993). To test this further, Leydig cells were isolated from aged rat testes and were found to produce significantly less testosterone in response to LH than cells from young rats (Chen et al., 2009). Because aged Leydig cells are less responsive to LH, this also results in reduced cAMP production in response to LH (Chen et al., 2002). This disruption in cAMP signaling is thought to be partially due to decreased LH receptor coupling to G_s protein, either through decreased mobility in the plasma membrane due to lipid cross-linking (peroxidation model) or through desensitization of the LH receptor due to persistent hormonal exposure (Midzak et al., 2009).

Aged Leydig cells are physiologically different from their younger counterparts. The steroidogenic capacity of Leydig cells is estimated to be reduced by about 50% with aging (Zirkin and Tenover, 2012). However, the reason for this reduction is not due to one single factor. In fact, aged Leydig cells have been shown to have deficiencies in LH receptor number cAMP production, StAR, TSPO, cholesterol transport, and the enzymes required for steroidogenesis in the mitochondria and smooth endoplasmic reticulum (Chen et al., 2009). Changes in these multiple components of the steroidogenic process have been supported by experimental evidence. In Brown Norway rats, reduction in the ability to produce testosterone is partially due to decreased expression of the steroidogenic enzymes (Midzak et al., 2009). In fact, both StAR and TSPO mRNA and protein levels have been shown to be diminished with age, as is cholesterol transport into the mitochondria (Culty et al., 2002; Midzak et al., 2009). Aged cells have also been shown to have a decreased response to LH, which also contributes to a reduced maximal testosterone synthesis in aged Leydig cells (Midzak et al., 2009). Aged Leydig cells also show increased COX-2 gene expression, which is known to inhibit steroidogenesis, possibly due to the increase in arachidonic acid metabolism by COX-2 (Chen et al., 2009).

Redox imbalance and oxidative stress could also play a role in age-related decreases in steroidogenesis (Zirkin and Tenover, 2012). Experimental evidence has shown that certain genes responsible for scavenging and/or repairing free radical-induced damage are down-regulated in aged cells. Some of these include Cu-Zn superoxide dismutase (SOD1), microsomal glutathione S-transferase (MGST1), and glutathione S-transferase (GSTM2) (Chen et al., 2009). Another theory supported by experimental evidence proposes that reactive oxygen species (ROS) derived from the mitochondrial electron transport chain and

steroidogenesis and/or macrophages could cause damage to cell membrane lipids and proteins of the Leydig cell by altering the redox environment that results in reduced LH signaling in aged cells (Chen et al., 2009). Because Leydig cells have an antioxidant network devoted to eliminating free radical species, as well as high levels of enzymes able to participate in electron transfer, it is thought that this network is vital for protection of cellular breakdown due to the presence of oxygen radicals (Midzak et al., 2009).

In order to elucidate the role of long term LH exposure in Leydig cells, a variety of experiments examining the relationship between LH stimulation and ROS formation were performed. Microarray analysis conducted on the Leydig cells of young adult rats stimulated by LH showed an up-regulation of genes involved in G protein-coupled receptor activation pathways. Many of these genes were involved in the stress response, including some genes known to be protective against cell death. These results suggested that LH stimulation might result in an increase of intracellular oxidative stress, which would subsequently cause cellular damage (Beattie et al., 2013). It was also seen that treatment of both MA-10 Leydig tumor cells and young adult rat primary Leydig cells saw an increase in ROS five minutes after treatment with LH, but this effect decreased forty-five minutes after stimulation. This effect was also seen in aged Leydig cells, but the time to peak ROS production was longer compared to young cells (Beattie et al., 2013). Incubation of MA-10 cells with LH also showed increases in DNA damage, but this damage could be repaired over time or suppressed when cells were treated with vitamin E, suggesting that the DNA damage was due to ROS generation in the cells. Together, these results indicate that Leydig cell stimulation by LH can induce increased ROS production and DNA damage in young and old cells, but the extent of these effects are greater in aged cells (Beattie et al., 2013).

TSPO and TSPO Drug Ligands

TSPO, originally named the peripheral benzodiazepine receptor, was discovered due to its ability to bind the benzodiazepine diazepam with high affinity (Papadopoulos and Miller, 2012). TSPO can also bind isoquinoline carboxamides (Giatzakis and Papadopoulos, 2004). First found in the kidney where radiolabeled benzodiazepine had binding sites, it was later confirmed to be present in most tissues of the body (Issop et al., 2013). Secretory and glandular tissues, especially cells that produce steroid hormones, are rich in TSPO whereas liver and brain tissue expresses low levels of TSPO (Giatzakis and Papadopoulos, 2004). The name TSPO was adopted in 2006 (Rupprecht et al., 2010). Localized in the outer mitochondrial membrane, it is a ubiquitous mitochondrial protein present in steroidogenic tissues including the brain, adrenal glands, and testes (Papadopoulos and Miller, 2012; Issop et al., 2013). There is a high degree of homology between different species in the coding region, suggesting that TSPO has been highly conserved throughout evolution (Giatzakis and Papadopoulos, 2004). For example, the promoter for TSPO is highly conserved between the mouse and rat genomes, but the promoter is only conserved within the first 150 base pairs when comparing mice with humans (Giatzakis and Papadopoulos, 2004). TSPO is a five transmembrane domain protein that is 169 amino acids, and its location in the outer mitochondrial membrane suggests it acts as a cholesterol channel to the inner mitochondrial membrane (Rupprecht et al., 2010; Papadopoulos and Miller, 2012; Girard et al., 2012). Human and rat genes for TSPO have been entirely sequenced, and the gene was found to be composed of four exons, of which exon 1 and half of exon 4 are untranslated (Giatzakis and Papadopoulos, 2004). The C-terminus of this protein contains a cholesterol-recognition

amino acid consensus (CRAC) domain where TSPO binds cholesterol (Papadopoulos and Miller, 2012; Issop et al., 2013).

TSPO plays a vital role in the body, a fact supported by the findings that functional inactivation of TSPO caused an early embryonic lethal phenotype in mice (Papadopoulos et al., 1997; Rupprecht et al., 2010). Expression of TSPO appears to be regulated by the pituitary gland, but may also be affected by IL-1, dopamine, serotonin, norepinephrine, ginkgolide B, TNF- α , and several peroxisome proliferators (Giatzakis and Papadopoulos, 2004). Function of TSPO throughout the body may be affected by tissue- and cell-specific composition of the mitochondrial membranes (Rupprecht et al., 2010). TSPO mRNA expression profiles found that TSPO mRNA levels are relatively high in the kidney, spleen, muscle, lung, adrenal gland, thymus, and stomach in mice. Intermediate expression levels were seen in the pancreas, uterus, prostate, heart, and testis, with low expression levels seen in the liver. Whereas TSPO protein levels seemed to be directly proportional to mRNA levels in adrenal glands, the liver, and the brain in this study, they also appeared to be inversely proportional in the kidney and testis. One reason for this could be that TSPO is potentially regulated at the transcriptional level in the adrenal glands, liver, and brain but postranscriptionally in the kidney and testis (Giatzakis and Papadopoulos, 2004). A comparison of TSPO mRNA and protein levels in Leydig cell MA-10 and adrenal cell Y1 steroid producing cell lines and non-steroidogenic mouse embryo fibroblasts showed that TSPO levels are highest in MA-10 cells, suggesting differential posttranscriptional regulation of TSPO (Giatzakis and Papadopoulos, 2004). TSPO has been found to be involved in a variety of cellular functions such as oxidative stress, cellular proliferation, programmed cell death, and possibly tumorigenesis (Giatzakis and Papadopoulos, 2004). While involved in

many cellular activities, the focus of this review will be on the role of TSPO in steroidogenesis.

Because the C-terminus of TSPO is exposed to the cytosol, it may play a vital role in cholesterol uptake and import into the inner mitochondrial membrane (Rupprecht et al., 2010). This ability to bind cholesterol and import it into the mitochondria may also cause TSPO to be involved in regulating mitochondrial membrane biogenesis (Rupprecht et al., 2010). The multifaceted role of TSPO in the body and its importance in steroidogenesis has made this protein a fascinating new focus of research on steroidogenic regulation in the body.

TSPO Ligands

Drug ligands of TSPO have been a major point of research into various aspects of steroid production throughout the body. Drug ligands with affinity for TSPO have been shown to increase steroid production in the adrenal gland, gonads, and brain (Papadopoulos and Miller, 2012). They act by stimulating steroidogenic cells to induce the translocation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Papadopoulos and Miller, 2012). In the testis, TSPO drug ligands have the potential to increase endogenous testosterone production in steroid hormone deficient males without having to introduce exogenous testosterone to the body. In the brain, TSPO ligands are promising because they can target multiple parts of the neurodegenerative process such as neuroinflammation, oxidative stress, mitochondrial dysfunction, and neuronal loss in addition to characteristic pathology of these diseases such as β -amyloid protein in Alzheimer's disease (Barron et al., 2013; Rupprecht et al., 2010). Ligands of TSPO have proved to reduce microglia and macrophage activation, as well as the production of inflammatory cytokines

(Rupprecht et al., 2010). By better understanding how TSPO interacts with cellular components of disease, researchers may be able to target aspects of disorders for therapeutic applications.

Cholesterol binds to the cytosolic C-terminus at the conserved CRAC domain of TSPO, whereas other TSPO ligands bind to a region in the amino terminus of the protein (Rupprecht et al., 2010). A variety of chemical compounds have been examined as potential mediators in the steroidogenic process. For example, TSPO ligands other than cholesterol have been an important target for research into neurodegenerative diseases. One such ligand, Etifoxine, has neuroregenerative effects likely mediated by TSPO and has the potential to serve as treatment for peripheral nerve injuries and axonal neuropathies (Girard et al., 2011).

Because many studies showed that TSPO drug ligands could increase steroid production in various models of steroidogenic processes, it was proposed that direct activation of TSPO through TSPO drug ligands might increase testosterone production in Leydig cells (Chung et al., 2013). To test this hypothesis, the effects of TSPO drug ligands Ro5-4864 (a benzodiazepine) and FGIN-1-27 (an indole acetamide) were examined by treating primary cells from young and old Brown Norway rats. Further, in vivo tests were performed in order to see if FGIN-1-27 could increase testosterone production in old animals to levels comparable to young rats. While both young and old primary Leydig cells responded to Ro5-498 and FGIN-1-27 with increasing TSPO drug ligand concentration, the testosterone production by aged and young cells to FGIN-1-27 was more dramatic. Also, when Leydig cells were incubated with both FGIN and LH (at a concentration of 0.1 ng/mL), significantly higher testosterone production was seen compared to primary cells incubated

with FGIN-1-27 alone. From these results, it was proposed that Ro5-498 and FGIN-1-27 stimulated cholesterol transport into the mitochondria through action by TSPO (Chung et al., 2013). For in vivo tests, young and aged rats received daily injections of FGIN-1-27 over a period of ten days. After this time frame, testosterone serum levels were measured. It was found that both young and aged rats had higher serum testosterone relative to controls in response to FGIN-1-27 treatment. Data also showed that old rats had serum testosterone levels as high as non-stimulated young rats. These results suggest that TSPO drug ligands can bypass cAMP and activate TSPO directly, thereby increasing testosterone formation in older cells (Chung et al., 2013). The therapeutic potential for these results is quite significant, as they indicate that activation of TSPO by TSPO ligands could be used for the gonadotropin-independent induction of testosterone formation in both primary and secondary hypogonadism (Chung et al., 2013).

Therapeutic Treatments for Reduced Steroid Production

Before treatment can be offered for reduced testosterone, there must be an agreed upon “cutoff” to label a man as testosterone deficient. This proves problematic as there are multiple terminologies used to describe the state of testosterone in the body. Free testosterone and albumin-bound testosterone are what is referred to as bioavailable testosterone, but bioavailable levels of testosterone actually decrease to a greater extent than total testosterone in the body due to increases in sex hormone-binding globulin levels with age (Zirkin and Tenover, 2012). Regardless, testosterone replacement may offer notable health benefits for older men and prevent future health problems (Zirkin and Tenover, 2012).

Potential Therapeutic Applications for TSPO in Other Systems

Much information about the steroidogenic process has been elucidated from studying the cellular mechanisms and components involved in this process. Because TSPO is known to play such an important role in the production of testosterone, TSPO drug ligands are thought to be potential therapeutic agents that can pharmacologically activate Leydig cells to produce testosterone and other steroid hormones as needed.

Therapeutic Potential for TSPO Outside of the Reproductive System

While TSPO is found throughout the body, its role in the brain is unique. The mechanisms of interaction between the components of steroidogenesis are different in neural cells compared to cells in the rest of the body. For example, the low level of P450_{scc} and its mRNA found in the brain and peripheral nervous system suggests regulation of P450_{scc} gene expression may be different in the brain compared to other steroidogenic tissues (Zhang et al., 1995). So while the basic biochemical mechanisms underlying steroidogenic action may be similar throughout different physiological systems in the body, genetic mechanisms controlling how steroidogenic components are expressed and controlled differ. The presence of TSPO in a variety of neurological injuries and disease has been well established. TSPO has been found to be a biomarker for inflammation, brain damage including traumatic brain injury, and neurodegeneration (Kreisl et al., 2013; Rupprecht et al., 2010). In many parts of the nervous system, changes in how TSPO is expressed are characteristic of disease. Expression of TSPO following injury or disease has been seen in both the central and peripheral nervous systems at lesion sites. In the peripheral nervous system, TSPO expression upregulation occurs after injury primarily in Schwann cells and macrophages, but

can also occur in neurons (Girard et al., 2011). Upregulation of TSPO in astrocytes and microglia due to lesions has a direct association with the degree of damage done to the tissue (Rupprecht et al., 2010). TSPO upregulation has also been implicated as a potential marker for degenerative changes in Alzheimer's disease, frontotemporal dementia, Multiple Sclerosis, Huntington's disease, Amyotrophic Lateral Sclerosis, and Parkinson's disease (Rupprecht et al., 2010). TSPO expression has also been examined as part of other types of brain related disorders. For example, TSPO was shown to be overexpressed in various cancers including brain tumors. Also, reduced TSPO expression was seen in psychiatric disorders such as generalized anxiety disorder, social anxiety disorder, PTSD, and panic disorder in the presence of adult separation anxiety disorder (Rupprecht et al., 2010). In addition, TSPO binding may be influenced by drugs given as pharmaceutical treatment for neuropsychiatric disorders. In both animal and cell models, the atypical antipsychotic clozapine was found to increase TSPO binding in steroidogenic cells and tissue, suggesting that TSPO could play a role in the modulation of steroidogenesis by clozapine (Danovich et al., 2008). However, animals treated with clozapine in this experiment showed an increase in TSPO binding in the hippocampus, hypothalamus, adrenals, and in the testes (Danovich et al., 2008). If TSPO ligands are to be used therapeutically, their effect on multiple systems in the body must be considered. While more research is necessary, these results point to the importance of TSPO in multiple neurological and neuropsychiatric disorders.

In the central nervous system, TSPO is expressed primarily in the microglia and reactive astrocytes whereas in the peripheral nervous system TSPO is expressed in macrophages and/or Schwann cells (Rupprecht et al. 2010). TSPO is thought to be involved in autocrine and paracrine signaling responses of glial cells due to disease and/or injury. It is

this principle that has led to the hypothesis that TSPO ligands can be of therapeutic potential similarly to how they may be used in the gonads (Rupprecht et al., 2010). TSPO ligands stimulated steroid hormone production in the testes of both young and old Brown Norway rats at equivalent levels, suggesting that these ligands can ameliorate the effects of aging in this steroidogenic system (Chung et al., 2013). If steroid production has been shown to be affected by TSPO ligands in the reproductive system, it stands that it may also be a target for manipulation in neurosteroid production as well.

TSPO has also been implicated in neuronal regenerative processes, and ligands of this protein have been investigated as potential therapeutic agents for enhancing neuroprotection, increasing axonal regeneration, and influencing inflammation (Rupprecht et al., 2010; Girard et al., 2011). It is thought that TSPO may play a role in the nerve repair process as TSPO expression returns to resting levels only when regeneration is completed following peripheral nerve injury (Lacor et al., 1999; Rupprecht et al., 2010). In regards to central nervous system diseases, TSPO upregulation and prevalence in a variety of disorders have led researchers to hypothesize that targeting TSPO could modify disease progression (Daugherty et al., 2013).

TSPO in Alzheimer's Disease

Alzheimer's disease (AD) research is another area in which TSPO ligands have been examined for their potential therapeutic properties. 3xTgAD mice serve as a rodent model for Alzheimer's disease, and were used in a study to examine if the TSPO ligand Ro5-498 could serve as a potential therapeutic target for this neurodegenerative disease (Barron et al., 2013). Because neurosteroid levels may be altered in Alzheimer's disease (Yue et al, 2005;

Rosario et al., 2011; Barron et al., 2013), it was thought that alteration of neurosteroid synthesis due to administration of Ro5-498 might have an effect on AD pathology. Ro5 treatment was found to attenuate β -amyloid protein accumulation in gonadectomized young-adult male mice and in aged 3xTgAD mice, decreased gliosis in young-adult gonadectomized 3xTgAD mice, and increased brain testosterone and progesterone levels in young gonadectomized 3xTgAD mice but not aged mice. There was also morphological evidence that Ro5-498 treatment decreased astrocytic and microglial activation in both young and aged 3xTgAD mice. Because anxiety may be an early indicator of AD in people with mild cognitive impairment, TSPO ligand affect on anxiety-related behavior was measured in 3xTgAD mice. Mice treated with Ro5-498 exhibited a trend toward decreased anxiety-related behavior (Barron et al., 2013). These findings suggest that TSPO ligands can have a versatile role in AD research, representing a marker for neuroinflammation, acting as a possible anti-inflammatory treatment, and having a therapeutic effect on clinical features of AD such as anxiety (Barron et al., 2013).

TSPO ligands have therapeutic potential because of their ability to influence neurosteroid production at the point of cholesterol uptake (Irwin and Brinton, 2014). This is important because neurosteroids have been shown to affect and sometimes reverse Alzheimer's disease pathology. Allopregnanolone is an endogenous neurosteroid that declines with age and also during the course of neurodegenerative disease. This neurosteroid has also been shown to promote neurogenesis, improve cognitive capabilities, and reduce AD pathology in mice (Irwin and Brinton, 2014). However, therapeutic treatment with neurosteroids poses some problems. Questions of the optimal treatment paradigm arise in order to be maximally efficacious at reducing AD pathology while also promoting

neurogenic changes, especially since high doses of allopregnanolone and chronic treatment have been shown to impair learning and memory capabilities (Irwin and Brinton, 2014). Also, the majority of neurosteroids are not well suited for oral delivery due to poor solubility properties and poor bioavailability caused by the fact that neurosteroids precipitate readily from aqueous environments. These non-optimal chemical properties have led researchers to investigate other means to administer allopregnanolone besides oral dosage (Irwin and Brinton, 2014). TSPO ligands may offer hope as a novel method to induce endogenous hormone synthesis of neurosteroids, and thus bypass the problems mentioned above while still reaping the benefits of increased steroid levels. By targeting the brain's regenerative system and intervening with AD progression early on through new pharmacological treatments, headway may be made in addressing the millions of people affected by this disease (Irwin and Brinton, 2014).

TSPO in Multiple Sclerosis

Various TSPO ligands have shown promise in the treatment of Multiple Sclerosis (MS). One such ligand, etifoxine, was originally designed to be an anxiolytic agent but was later determined to have a strong affinity for TSPO (Daugherty et al., 2013). Because MS patients show a drop in neurosteroid levels and treatment with neurosteroids has been shown to lead to a partial rescue in a mouse model of MS, etifoxine was studied in order to determine if it had protective and regenerative effects on inflammatory demyelination in a mouse model of MS. Etifoxine treatment was shown to promote steroidogenesis and possibly inhibit peripheral immune cells in this model. Etifoxine-treated mice also showed a delay in onset of the first signs of clinical symptoms and a decrease in the peak of clinical scores in this model of MS. By modulating the activity of TSPO with etifoxine, less

peripheral immune cell infiltration of the spinal cord was observed, as well as increased oligodendroglial regeneration after inflammatory demyelination in the mouse model of MS. Together, these results suggest that etifoxine is both protective and promotes recovery in a mouse model of MS (Daugherty et al., 2013).

Another study conducted by Harberts et al. (2013) examined the use of a newly synthesized TSPO ligand, PBR28, in an *in vitro* radioligand binding assay to quantify peripheral TSPO in peripheral blood mononuclear cells from MS patients and healthy controls. PBR28 had already been shown to correlate with active demyelinating lesions found during MRI in MS patients. Patients with MS were found to have a significantly lower amount of peripheral TSPO compared to the healthy control group. This finding in the PNS is in contrast to damage in the CNS in which TSPO is upregulated in response to disease state. This reduction in peripheral TSPO expression may be due to sequestration of macrophages expressing high levels of TSPO in the tissue, and can thus be explored as a potential peripheral biomarker of MS (Harberts et al., 2013).

TSPO in Neuroimaging

There has been an increased interest in the use of TSPO ligands as neuroimaging agents in recent literature. Radiolabeled ligands have been investigated as diagnostic tools and markers for the state and progression of neuronal injury and neurodegenerative disease (Rupprecht et al., 2010). Because TSPO upregulation in neuronal cells is associated with the degree of damage in those cells, TSPO imaging can be useful when assessing brain lesions with pathogenic heterogeneity such as following strokes (Rupprecht et al., 2010). TSPO binding has been frequently examined with the use of positron emission tomography (PET)

in recent studies. One study by Kreisl et al. (2013) used the radioligand ^{11}C -PBR28 in an attempt to determine if TSPO binding was different between groups of patients with Alzheimer's disease, mild cognitive impairment, and healthy older controls. Then, TSPO binding was analyzed to see if it corresponded with neuropsychological measures, grey matter volume, ^{11}C -Pittsburg Compound B binding (PIB), or in age of onset. Differences in binding was demonstrated through the different experimental groups, with AD patients showing greater ^{11}C -PBR28 binding in cortical brain regions known to be affected by AD pathology than controls, but this effect was not seen in patients with mild cognitive impairment (MCI). ^{11}C -PBR28 binding was also found to be inversely correlated with performance on the neuropsychological battery of tests as well as with grey matter volume. Interestingly, ^{11}C -PBR28 binding (particularly in the parietal cortex) was associated with earlier age of onset. ^{11}C -PBR28 binding indicated the presence of neuroinflammation, and so increased ^{11}C -PBR28 binding to TSPO after conversion of MCI to AD correlated with greater neuroinflammation and worsened disease progression. This suggests that ^{11}C -PBR28 can be used to measure increased TSPO expression by activated microglia, which occurs after the conversion between disease states. The differences in ^{11}C -PBR28 binding in early onset compared to late onset AD patients led to the hypothesis by the authors that microglial activation may be the driving pathogenic factor in early onset AD as opposed to amyloid burden, and might offer an explanation as to why early onset patients display more severe executive and visuospatial deficits compared to older onset individuals. These results also show that the radioligand ^{11}C -PBR28 may be utilized in longitudinal studies to mark the conversion from MCI to AD, or could be used to assess the response to experimental treatments of AD. However, although this study did not detect microglial

activation in subjects with MCI, this could just be due to the fact that ^{11}C -PBR28 is not sensitive enough to detect TSPO density in these patients. (Kreisl et al., 2013).

Another study by Yasuno et al. (2012), suggests the potential for TSPO binding measured by PET imaging as a way to monitor neurodegenerative disease progression. TSPO binding has been shown to reflect activated microglia, which also serves as a predictive biomarker of conversion from mild cognitive impairment to dementia (Yasuno et al., 2012). This study used the TSPO ligand [^{11}C]Daa1106 to measure TSPO binding in mild cognitive impairment, Alzheimer's disease, and age-matched control groups. TSPO was quantified by binding potential, and a portion of the MCI group was followed for five years after the initial PET scan to monitor disease progression. Strikingly, [^{11}C]Daa1106 binding to TSPO was increased significantly in widespread areas in the MCI groups compared to controls, although there was no significant difference between MCI and AD patients in all measured regions. However, the widespread binding of [^{11}C]Daa1106 in MCI and AD patients due to reactive microglia and astrocytes was not linked spatially to β -amyloid and tau pathology. In addition, MCI patients with [^{11}C]Daa1106 binding values higher than the control mean by +0.5 standard deviations developed dementia within five years of the original imaging studies. These results suggest that microglial activation as indicated by higher [^{11}C]Daa1106 binding can occur before the clinical onset of symptoms of dementia, and could be a predictive biomarker of early stage disease. This offers promise in being able to recognize MCI patients at a higher risk of conversion to dementia by TSPO imaging (Yasuno et al., 2012).

Conclusions

While much is known about the steroidogenic process in the body, there is still much to be learned. Steroid production is a finely tuned process that proceeds in a stepwise manner involving many different cellular components. Because there are so many steps involved, there are also many ways in which those cellular components can cause problems. Alternatively, there are also many ways in which those same cellular components can be manipulated in order to ameliorate deleterious effects. Many of these have been outlined in the above review. COX-2 inhibitors and TSPO drug ligands are of particular interest regarding their role in testosterone production in the aging Leydig cell. There is much therapeutic potential for these two pharmacological treatments in the steroidogenic process. Particularly in the male reproductive system, COX-2 inhibitors and TSPO drug ligands have the potential to offer an alternative pharmacological treatment for increasing testosterone synthesis in aging males. As can be seen through the extensive data on the potential use of TSPO drug ligands in the neurological system, the therapeutic applications of these drugs extend far beyond just the reproductive system.

**Effects of COX-2 Inhibitors and Translocator Protein Drug Ligands on Progesterone
Production by Mouse Leydig Tumor Cells**

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INTRODUCTION

Reduced serum testosterone (T), or hypogonadism, is common in aging men. Approximately 20-50% of men over age 60 are reported to have serum T levels significantly below those of young men (age 20-30 years) (Harman et al., 2001; Surampudi et al, 2012). Adult Leydig cells synthesize T under the control of luteinizing hormone (LH) from the pituitary gland. Age-related decline in serum T levels in many hypogonadal men, however, is not in response to reduced LH from the pituitary gland (secondary hypogonadism), but rather a consequence of Leydig cells becoming less responsive to LH (Surampudi et al., 2012; Zirkin and Tenover 2012). This is referred to as primary hypogonadism. Whether in aging or young men, reduced serum T has been linked to a number of metabolic and quality-of-life changes, including decreased lean body mass and bone mineral density, decreased muscle mass and strength, adiposity, cardiovascular disorders, decreased libido and sexual function, as well as altered mood and fatigue (Surampudi et al., 2012).

One way to treat the symptoms of low T is through exogenous T replacement. Exogenous T preparations can be used to raise serum T levels and treat the symptoms of low T. The T preparations in use to achieve this are injections, scrotal and nonscrotal transdermal patches, and oral, buccal and gel preparations. Testosterone injections and testosterone gel preparations are two of the most commonly used replacement methods in the United States (Surampudi et al., 2012). With injections, serum T levels can vary, which requires T levels to be measured and sometimes adjusted between injections. T administered by gels and other transdermal methods produce more constant T concentrations, but these methods have the potential for unwanted T transfer via skin contact (Surampudi et al., 2012). Testosterone replacement therapy does come with the risk of potentially serious side effects such as

cardiovascular complications, sleep apnea, polycythemia and prostatic disease (Surampudi et al., 2012). Moreover, the administration of exogenous T by any means can suppress gonadotropin release from the pituitary, leading to reduced intratesticular testosterone and consequently reduced sperm production. Thus, the exogenous administration of T to ameliorate hypogonadism could affect fertility (Surampudi et al., 2012).

Increasing serum (and intratesticular) T by stimulating the Leydig cells themselves rather than administering exogenous T could have great advantages. Increasing Leydig cell T production should result in the physiological regulation of T by its negative feedback on LH. This is in contrast to essentially flooding the system with T, as with exogenous T administration. This method is advantageous because it does not pose the risk of some side effects seen with exogenous T administration. This approach should elicit fluctuations in T levels in response to negative feedback of T on LH, as occurs normally. There would not be the potential for T transfer to others via contact. Fertility should be preserved, not suppressed, because the hypothalamic-pituitary axis should not be shut down as with exogenous T. Indeed, the local stimulation of Leydig cell T production might actually enhance spermatogenesis because intratesticular T levels should increase, not decrease, and because the Leydig cells produce more than just testosterone.

How might the Leydig cells in individuals with primary hypogonadism be stimulated to produce T? In Leydig cells, LH binds to and activates G protein-coupled receptors, resulting in the activation of adenylyl cyclase and thus in increased cAMP formation. The acute stimulation of Leydig cells by LH results in cholesterol transfer from intracellular stores into the mitochondria, which is the rate-limiting step in steroid biosynthesis. This is followed by the conversion of cholesterol to pregnenolone by the cholesterol side-chain

cleavage cytochrome P450 enzyme (CYP11A1), located on the matrix side of the inner mitochondrial membrane. Pregnenolone is then converted to testosterone in the smooth endoplasmic reticulum (Midzak et al., 2009). Translocator protein (TSPO) is part of a complex of proteins residing in both the inner and outer mitochondrial membranes involved in this cholesterol transport (Rupprecht et al., 2010). The C-terminus of this protein contains a cholesterol-recognition amino acid consensus (CRAC) domain where TSPO binds cholesterol (Papadopoulos and Miller, 2012; Issop et al., 2013). Numerous previous studies have shown that TSPO drug ligands are able to stimulate steroid formation in steroidogenic cells (Chung et al., 2013; Papadopoulos and Miller, 2012).

Previous studies demonstrated that the direct, pharmacological activation of TSPO with the TSPO drug ligand FGIN-1-27 (*N,N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide) increased T production by young and aged Leydig cells (Chung et al., 2013). The stimulatory effect of FGIN-1-27 was abolished by the TSPO cholesterol recognition/interaction amino acid consensus (CRAC) domain inhibitor 3,17,19-androsten-5-triol (19-Atritol). FGIN-1-27 also had effects when administered in vivo. Thus, administering FGIN-1-27 to aged rats elevated serum T to the level of young rats. These studies showed that the pharmacologic activation of TSPO could increase T production and led to our decision to evaluate the potential use of TSPO drug ligands as one means to induce T formation by steroid-forming cells.

Another class of chemical compound, COX-2 inhibitors, also has been examined as a means by which to increase T production by Leydig cells. LH acts principally through a cAMP dependent pathway to simulate cholesterol transport and steroidogenic enzymes (Chen et al., 2007). However, in addition to cAMP synthesis and signaling, LH also induces the

release of intracellular arachidonic acid, which is metabolized by lipoxygenase, epoxygenase, or cyclooxygenase stores in the cell (Midzak et al., 2009). It is thought that expression of cyclooxygenase 2 (COX-2) in the Leydig cells plays a role in the declining levels of testosterone seen in aging Leydig cells (Chen et al., 2007). COX-2 may in fact suppress steroidogenesis since inhibition of COX-2 was shown to increase StAR and progesterone production in MA-10 Leydig cells stimulated with dbcAMP (Wang et al., 2003). The observation that inhibition of COX-2 activity increases the sensitivity of steroidogenesis to cAMP stimulation in MA-10 Leydig cells, and lowers the concentration of cAMP necessary for maximal steroid production (Wang et al., 2003), resulted in our decision to evaluate the potential use of COX-2 inhibitors and TSPO ligand combinations on steroid formation.

In sum, two cellular components, COX-2 inhibitors and TSPO drug ligands, are of particular interest regarding their role in steroid formation. The rationale for conducting studies of combinations of the two is that separately or together, COX-2 inhibitors and TSPO drug ligands have the potential to offer an alternative pharmacological treatment to combat hypogonadism in young and aging males.

MATERIALS AND METHODS

Reagents

Apigenin, indomethacin (indo), dbcAMP, Ro5-4864, FGIN-1-27, Waymouth MB752/1 medium, horse serum, and Hanks' Balanced Salt Solution were obtained from Sigma-Aldrich (St. Louis, Missouri). M-199 was from Gibco BRL (Grand Island, New York). Progesterone antibodies were obtained from MP Biomedical (Solon, Ohio). Bovine LH (USDA-bLH-B6) was provided by the US Department of Agriculture Animal Hormone Program (Beltsville, Maryland). NS398 was from Cayman Chemical (Ann Arbor, MI). 5,5-Dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl) phenyl-2((5H)-furanone (DFU) was kindly provided by Dr. Denis Riendeau from Merck Frosst Canada Ltd. (Kirkland, Quebec, Canada).

MA-10 Cell Culture

MA-10 mouse Leydig tumor cells were a gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA). The cells were grown in Waymouth's MB752/1 medium containing 15% horse serum and 5% CO₂ at 34° (Chen et al., 2010). Cells were grown in 24 well plates, and treated when they reached 70-80% confluence. When cells were undergoing treatment with LH, dbcAMP, COX-2 inhibitors, or TSPO drug ligands, serum-free M199 medium was used. The medium was frozen for progesterone assay and the cell plate was frozen for future protein assays.

MA-10 Cell Treatment with LH, dbcAMP, and COX-2 Inhibitors

MA-10 cells were grown on a 24 well plate in Waymouth MB752/1 medium with 15% FBS until the cells were 70-80% confluent. Once the cells reached this point, they were

treated with one of four different COX-2 inhibitors (Apigenin, DFU, NS398, or Indo). Serum-free M-199 medium containing 0.1% BSA was used during treatment. The different COX-2 inhibitors were dissolved in DMSO and then diluted in the culture medium with the final concentration of DMSO in the medium always being less than 0.2%. Cells were treated with five different concentrations of COX-2 inhibitors: 0.1 μ M, 0.33 μ M, 1.0 μ M, 3.3 μ M, or 10 μ M. Cells were incubated for 30 min at 34° C with the COX-2 inhibitors. After this period, cells were treated with either culture medium alone (control), LH or dbcAMP, and incubated for 2 hr at 34° C with the continued presence of the inhibitors. After this incubation, medium was collected from each well and immediately frozen at -20°C for progesterone assay. Progesterone levels were assayed by radioimmunoassay (RIA).

MA-10 Cell Combination Treatment with COX-2 Inhibitors and TSPO Drug Ligands

For the combination treatment, MA-10 cells were treated with one of two different COX-2 inhibitors, DFU or Indo. Cells were also treated with one of two different TSPO drug ligands, Ro5-4864 or FGIN-1-27. The procedure was the same as described above, with the cells incubated with the COX-2 inhibitor and TSPO drug ligand combination for 30 min before the addition of LH or dbcAMP. Cells were treated with COX-2 inhibitors alone, TSPO drug ligands alone, or in combination. Cells that only received either a COX-2 inhibitor or TSPO drug ligand were given an additional amount of DMSO in order to balance out well volumes. Cells were then incubated for 30 min at 34° C. After this 30 min incubation, cells were treated with either culture medium, LH, or dbcAMP and incubated for 2 hr at 34° C with the continued presence of the inhibitors. After this incubation, medium was collected from each well and immediately frozen for later progesterone assay by RIA. Cells were also frozen and stored at -80°C to be used for the MTT assay.

MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to assess cell viability (Mossman, 1983; Chen et al., 2010). After treatment with COX-2 inhibitors and TSPO drug ligands for two hours, cells were incubated with M199 medium containing MTT (0.5mg/ml) for one hour. In the viable cells, the MTT can be converted into blue formazan. The medium was then removed and the plate was frozen at -80°C to be tested at a later date. When the assay was to be performed, the reduced formazan in each well was dissolved with 200 µL acidified (0.04 N HCl) isopropanol at room temperature for 30 minutes. The solution from each well was then transferred to a new 96-well plate and read by a DTX800 Multimode Detector (Beckman, Coulter, Inc., Fullerton, CA) at 562 nm wavelength. Cells from three different experiments were analyzed for each treatment with COX-2 inhibitors, TSPO drug ligands, and 0.5 ng/mL LH stimulation.

Statistical Analysis

Data are expressed as Mean +/- standard error of the mean (SEM). One-way ANOVA was used to evaluate group means. If differences between groups were detected by ANOVA ($p < 0.05$), then differences between individual groups were determined by the Student-Neuman-Kuels test. The tests were done by SigmaStat software for Windows (Version 2.03).

RESULTS

COX-2 inhibitors and TSPO drug ligands have been shown to have stimulatory effects on steroid production (Chung et al., 2013; Wang et al., 2003). The primary objective of this Master of Science research was to determine whether a combination treatment of COX-2 inhibitors and TSPO drug ligands would have effects that differ from their individual effects on steroid production by MA-10 Leydig cells. To this end, MA-10 cells were cultured with inhibitor and/or ligand in the presence of LH or dbcAMP, and their separate and combined effects on the production of progesterone were measured.

We first determined the effect of increasing concentrations of LH alone on progesterone production by MA-10 Leydig cells. For this study, cells were cultured for 2 hours with increasing concentrations of LH (0-100 ng/ml), and progesterone concentration in the media was measured by RIA. As seen in Figure 1, LH at 0.01 ng/ml had little effect. A significant increase in progesterone production was seen in response to 0.1 ng/ml. At concentrations between 1 and 100 ng/ml, progesterone was maximally stimulated.

We next assessed the effect of increasing concentrations of COX-2 inhibitors on progesterone production. The objective was to determine at least preliminarily the relative effects of the inhibitors on progesterone formation. MA-10 cells were incubated for 30 minutes with apigenin, DFU, NS398, or indomethacin (Indo), at concentrations from 0.1 μ M to 10 μ M. Then, LH at a concentration of 0.1 ng/ml (Fig. 2A) or 1.0 ng/ml (Fig. 2B) was added, and the cells were incubated for an additional two hours. After treatment, media samples were collected and progesterone levels were measured. The studies were repeated twice, with averages shown. As seen in Figure 2A, the four COX-2 inhibitors did not have

dramatically different effects from each other on progesterone production in response to 0.1 ng/ml LH, regardless of dose. Rather, progesterone levels were relatively low across all concentrations of COX-2 inhibitors tested. When tested at an LH concentration of 1.0 ng/mL, a concentration at which progesterone levels were considerably higher, the four COX-2 inhibitors again produced similar effects across all five concentrations tested. We noted that the highest progesterone production was seen when DFU was used at a concentration of 10 μ M (Fig. 2B).

Figures 3A-D compare the dose-dependent effects on progesterone production in response to each of the four COX-2 inhibitors (apigenin, Fig. 3A; DFU, Fig. 3B; NS398, Fig. 3C; and Indo, Fig. 3D). As above, the cells were treated with COX-2 inhibitor, and then LH was added at 0.1 or 1 ng/ml. In each case, progesterone levels were significantly greater when LH was at a concentration of 1.0 ng/mL. However, it is apparent there was little difference among the five different doses of COX-2 inhibitors whether LH was either 0.1 or 1 ng/ml. This confirmed that the COX-2 inhibitors elicited similar effects to one another.

Cyclic AMP is produced in response to LH/receptor binding and is integrally involved in cholesterol translocation from the cytosol to the inner mitochondrial membrane, the rate-determining step of steroid formation (Midzak et al., 2009). Previous experiments have shown that dbcAMP stimulation can increase steroidogenesis (Chen et al., 2007). We conducted a series of studies in which MA-10 cells were incubated with increasing concentrations of each of the four COX-2 inhibitors, but with stimulation by dbcAMP rather than with LH. As with the LH regimen, cells were incubated with a COX-2 inhibitor for 30 minutes, and then dbcAMP was added at a concentration of 0.1 mM (Fig. 4A) or 0.05 mM (Fig. 4B) for 2 hours. As seen in Figure 4A, when the cells were stimulated with 10 μ M

apigenin or DFU along with 0.1 mM dbcAMP, there were significant, 4-5-fold increases in progesterone production in comparison to stimulation with NS398 or Indo at that same concentration. At lower concentrations (0.1 – 3.3 μ M), there was little difference in the stimulation of progesterone production by the four COX-2 inhibitors. With 0.05 mM dbcAMP (Fig. 4b), progesterone production was again very high with DFU at 10 μ M, though not with apigenin. At lower concentrations (0.1 μ M, 0.33 μ M and 1 μ M), Indo stimulation also resulted in relatively high progesterone production.

From these results, which differed somewhat from those obtained with LH, we proceeded to examine the effects of TSPO drug ligands with DFU or Indo co-stimulation. Our objective was to determine whether using COX-2 inhibitors and TSPO drug ligands in combination had a different outcome than using the inhibitors and ligands separately. . The TSPO drug ligands used were Ro5-4864 (Ro5) and FGIN-1-27 (FGIN). The treatments that were used are shown in Figure 5. For these studies, cells were incubated with COX-2 inhibitor, TSPO drug ligand, or a combination of the two for 30 min. Then LH or dbcAMP was added, the cells were incubated for an additional 2 hours, and progesterone in the media was measured.

Mean progesterone levels for each treatment stimulated with LH can be seen in Figures 6A-6D. With 0.1 ng/ml LH (Fig. 6A), progesterone production was comparable by cells treated with the COX-2 inhibitors DFU and Indo. Stimulation by the TSPO drug ligand Ro5 resulted in progesterone production at levels that were comparable to those with DFU or Indo. Progesterone levels in response to the two TSPO drug ligands differed; stimulation with FGIN resulted in progesterone production that was about half that of Ro5. Progesterone production by combinations of Ro5+DFU and Ro5+Indo were not different from production with the COX-2 ligands or TSPO drug ligands alone. FGIN + DFU resulted in progesterone

production that was lower than, though not significantly different from, FGIN alone. But FGIN + Indo resulted in progesterone levels that were lower than Indo alone. There was no statistically significant difference between the different treatment regimens ($P>0.05$). However, standard error bars were not expressed for FGIN + DFU treatment ($n=2$), so statistical analysis does not include this treatment. As seen in Figures 6B and 6C, comparable results were seen when LH stimulation was at 0.2 ng/ml and 0.5 ng/ml, respectively. There was no statistically significant difference between treatment regimens when 0.2 ng/mL LH stimulation was used ($P>0.05$). However, there were statistically significant differences observed when 0.5 ng/mL LH stimulation was used ($P<0.05$). In Figure 6C, statistically different groups are represented with different letters. The most striking differences were observed when Ro5 was used alone or in combination with COX-2 inhibitors. Ro5 + Indo treatment, Ro5 + DFU treatment, and Ro5 treatment alone were not found to be statistically different from each other, but these three regimens resulted in the highest mean progesterone levels. However, Ro5 + Indo treatment was statistically different from Indo treatment alone. Ro5 + DFU treatment was found to be statistically different from all treatments except Ro5 alone, Indo alone, and Ro5 + Indo. In addition, Ro5 treatment alone was not statistically different from Indo treatment alone. The results obtained were not attributable to toxic effects of the treatments. Cell viability after treatment with LH (0.5 ng/ml) with COX-2 inhibitors and TSPO drug ligands either singly or together, was determined with the MTT assay. A maximally stimulating dose of 10 ng/mL LH was also tested. As can be seen in Figure 7, cell viability was not affected by any of the treatments and there was no statistical difference seen between different treatments ($P>0.05$).

Finally, we wished to assess whether using COX-2 inhibitors and TSPO drug ligands in combination had a different outcome than using the inhibitors and ligands separately, but in experiments in which the cells were stimulated with dbcAMP rather than with LH. The results are shown in Figure 8, and groups that are not statistically different from each other are represented by the same letter. The three regimens with the highest mean progesterone levels were 5 μ M Indo alone, 10 μ M Indo alone, and Ro5 + 5 μ M Indo. These three treatments were not statistically different from each other. However, treatment with 10 μ M Indo alone had mean progesterone levels slightly lower than the other two treatments, and this regimen was not statistically different from treatment with Ro5 + DFU, Ro5 + 10 μ M Indo, FGIN + DFU, or FGIN + 5 μ M Indo.

Indo treatment seemed to be eliciting higher mean progesterone levels with stimulation of both LH and dbcAMP, so we wanted to examine the effects of Indo on MA-10 cell steroid production in more detail. Because earlier experiments consistently showed promise for increasing progesterone production with Indo treatment, we decided to test Indo at a higher concentration than was done previously. In order to do this, MA-10 cells were treated with increasing concentrations of Indo. MA-10 cells were cultured in a 24 well plate and treated with Indo concentrations ranging from 0.5 μ M to 50 μ M with either LH or dbcAMP stimulation. Cells were incubated with Indo for 30 minutes, and then given LH at a concentration of 0.5 ng/mL or dbcAMP at a concentration of 0.1 mM. Cells were then incubated for two hours and medium samples were collected and frozen. Progesterone levels were measured by RIA. As with our previous experiments, mean progesterone levels were consistently higher when cells were stimulated with dbcAMP compared to those stimulated with LH (Fig. 9). Treatments that were not found to be statistically different from each other

are represented by the same letter. Progesterone levels increased when the concentration of Indo increased, but declined when 50 μ M Indo and dbcAMP stimulation were used, signifying possible toxic effects at this high concentration. However, treatments with dbcAMP and Indo at a concentration of 1.0 μ M, 5 μ M, 10 μ M, and 50 μ M were not statistically different from each other. All Indo treatments with LH were found to not be statistically different, except for LH treatment with 50 μ M Indo. Treatment with 50 μ M Indo and LH was not found to be statistically different from treatment with dbcAMP and Indo concentrations at 0.5 μ M, 1.0 μ M, 5.0 μ M, and 50 μ M.

DISCUSSION

COX-2 inhibitors and TSPO drug ligands each have been shown to have stimulatory effects on steroid production (Chung et al., 2013; Wang et al., 2003). TSPO drug ligands accomplish this by acting at the level of the TSPO component of the transduceosome to increase steroid production (Papadopoulos and Miller, 2012), whereas COX-2 inhibitors are thought to block the inhibitory action of COX-2 on Leydig cell steroid production (Wang et al., 2003). While these principles have been illustrated by various experiments separately, there is a lack of data examining if a combination treatment of both TSPO drug ligands and COX-2 inhibitors can have additive or synergistic effects on steroid production in the Leydig cell. To begin to explore this possibility, we first wished to identify COX-2 inhibitors to use in conjunction with the two TSPO drug ligands, FGIN-1-27 and Ro5-4864, found by Chung et al. (2013) to increase steroid hormone production in primary Leydig cells. Both ligands were shown to increase testosterone production through direct activation of TSPO. The results from this experiment led us to use both FGIN-1-27 and Ro5-4864 in our experimental paradigm.

Because LH is necessary for stimulation of progesterone production by MA-10 cells, we first sought to determine which LH concentrations would be best to use in experiments with COX-2 inhibitors and TSPO drug ligands. Our goal was to find the lowest concentration of LH that can produce the greatest increase in progesterone production relative to levels produced by a maximally stimulating dose of LH (100 ng/mL). Little effect was found at an LH concentration of 0.01 ng/ml. Approximately half maximal stimulation was found at 0.1 ng/ml, and maximal stimulation at 0.1 – 1.0 ng/ml. From these results, most experiments utilized LH at concentrations between 0.1 – 1.0 ng/ml. In an initial series of studies, the

effect of four different COX-2 inhibitors on progesterone production by MA-10 cells was determined. For these studies, MA-10 cells were cultured in a 24 well plate and treated with a combination of LH and increasing concentrations of the COX-2 inhibitors Apigenin, DFU, NS398, or indomethacin (Indo). Surprisingly, we found that the four COX-2 inhibitors had similar effects across all concentrations when examined in conjunction with LH stimulation, though with DFU and Indo showing slightly higher mean progesterone production at particular concentrations. This was true whether LH was used at 0.1 or 1.0 ng/l.

Previous studies of COX-2 inhibitor effects had utilized MA-10 cells stimulated by dbcAMP, which bypasses the LH/LH receptor G protein coupling (Wang et al., 2003). Consequently, we repeated our studies of COX-2 inhibitors, but with dbcAMP stimulation. Two concentrations of dbcAMP were used, 0.1 or 0.05 mM. When tested at concentrations of 1 μ M and 3.3 μ M and stimulated with 0.1 mM dbcAMP, DFU and Indo produced higher mean progesterone levels compared to the other COX-2 inhibitors. However at 10 μ M concentration, Apigenin and DFU had extremely high mean progesterone levels compared to the other COX-2 inhibitors. The same experiment then was conducted with the lower dbcAMP concentration (0.05 mM). DFU and Indo were the two COX-2 inhibitors shown to increase mean progesterone production compared to the other inhibitors tested across all concentrations.

We then conducted studies designed to determine whether treating MA-10 cells with combinations of COX-2 inhibitor and TSPO drug ligand would affect MA-10 cell progesterone production differently than when the cells were treated with COX-2 inhibitor or TSPO drug ligand separately. The studies were conducted with both LH and dbcAMP. MA-10 cells were cultured in a 24 well plate and treated with a combination of LH or dbcAMP,

and COX-2 inhibitors plus TSPO drug ligands. The COX-2 inhibitors used were DFU or Indo, and the TSPO drug ligands used were FGIN or Ro5. MA-10 cells were treated with LH at concentrations of 0.1 – 0.5 ng/ml. While there was increased steroid production when cells were treated with Ro5 or Ro5 + Indo at 0.1 ng/mL LH stimulation, there were no statistical differences seen across all treatment regimens. No statistical differences were observed between treatments when tested with 0.2 ng/mL LH stimulation. With 0.5 ng/mL LH, there were statistical differences observed between treatments. The most notable differences were observed when Ro5 was used alone or in combination with COX-2 inhibitors. Ro5 + Indo treatment, Ro5 + DFU treatment, and Ro5 treatment alone resulted in the highest mean progesterone levels and were not found to be statistically different from each other. Cell viability was examined in cells stimulated with 0.5 ng/mL LH by MTT assay. There was no statistically significant difference in cell viability across the various treatment regimens, lending credibility to the data.

In order to examine these differences in progesterone production in response to a combination of COX-2 inhibitor and TSPO drug ligand further, we elected to determine if stimulation with dbcAMP would also exhibit this same pattern. The highest mean progesterone levels were seen with 5 μ M Indo alone, 10 μ M Indo alone, and Ro5 + 5 μ M Indo, and these three treatments were not statistically different from each other.

When the effects of Indo treatment at various concentrations was tested with either LH or dbcAMP stimulation, cells stimulated with dbcAMP resulted in higher mean progesterone levels. Treatments with dbcAMP and Indo at a concentration of 1.0 μ M, 5 μ M, 10 μ M, and 50 μ M were not statistically different from each other. All Indo treatments with LH were not statistically different, except for LH treatment with 50 μ M Indo. Treatment

with LH and 50 μ M Indo was not found to be statistically different from treatment with dbcAMP and Indo concentrations at 0.5 μ M, 1.0 μ M, 5.0 μ M, and 50 μ M.

The hypothesis upon which these studies were based was that a combination treatment of COX-2 inhibitors and TSPO drug ligands would have a different effect on progesterone production, whether additive or synergistic, than either alone. This did not prove to be the case. Indeed, our results showed that the combination treatment sometimes decreases progesterone production, as when cells were treated with FGIN alone in comparison to FGIN plus DFU or FGIN plus Indo. The results obtained apparently were not a consequence of cell viability differences, though further studies of different combinations of COX-2 inhibitor and TSPO drug ligand must be conducted. Thus, although the mechanisms that are involved in how COX-2 inhibitors and TSPO drug ligands stimulate steroid formation differ, the two together have no greater effect than either alone when used with MA-10 cells. The possibility of additive or synergistic effects has not been assessed using primary cells or in vivo.

With regard to the COX-2 studies, it should be noted that much of the past work involving MA-10 cells was done using dbcAMP, and this work formed our rationale for using dbcAMP in our experiments. There were differences in the effects of COX-2 inhibitors in particular when MA-10 cells were stimulated with dbcAMP compared to LH. The results suggest that the effects of COX-2 inhibitors may only be present when dbcAMP is used. cAMP activates many cellular pathways in addition to the steroidogenic pathway, perhaps including those that could affect steroidogenesis. Thus the COX-2 pathway may not be relevant under normal LH stimulation, but may become significant when dbcAMP activates this potential new pathway. The differences in progesterone production may in fact

be a side effect of this activated pathway. If this hypothesis is correct, it leads to the question of whether dbcAMP should be used instead of LH when studying MA-10 cells, as has been done in past studies. While the mechanisms behind the differences between progesterone production in MA-10 cells stimulated with LH versus dbcAMP is unclear, future testing is needed in order to examine if the proposed combination treatment with COX-2 inhibitors (specifically Indo) and TSPO drug ligands is a viable option for increasing steroid production in aged or hypogonadal individuals.

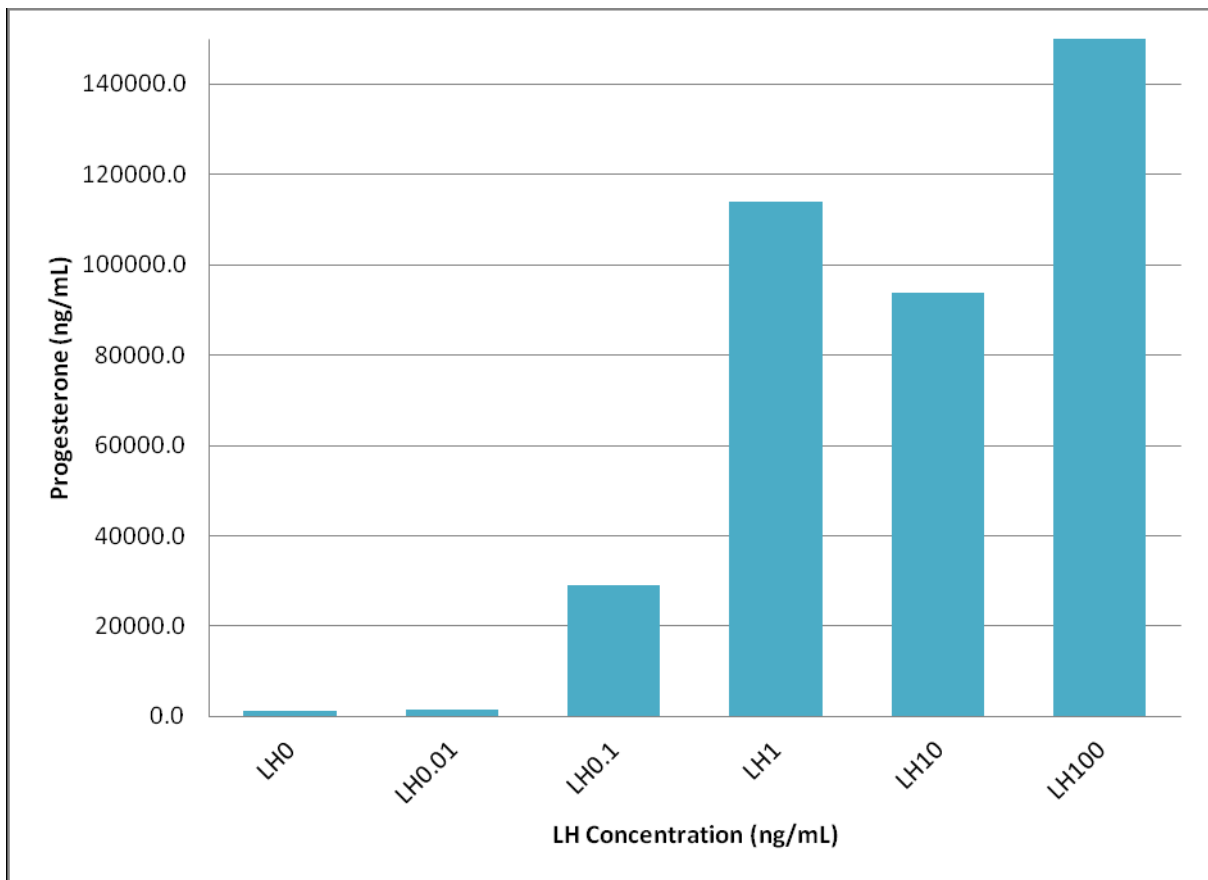


Figure 1: Progesterone levels produced by MA-10 cells in response to increasing LH concentrations. Progesterone was measured with radioimmunoassay.

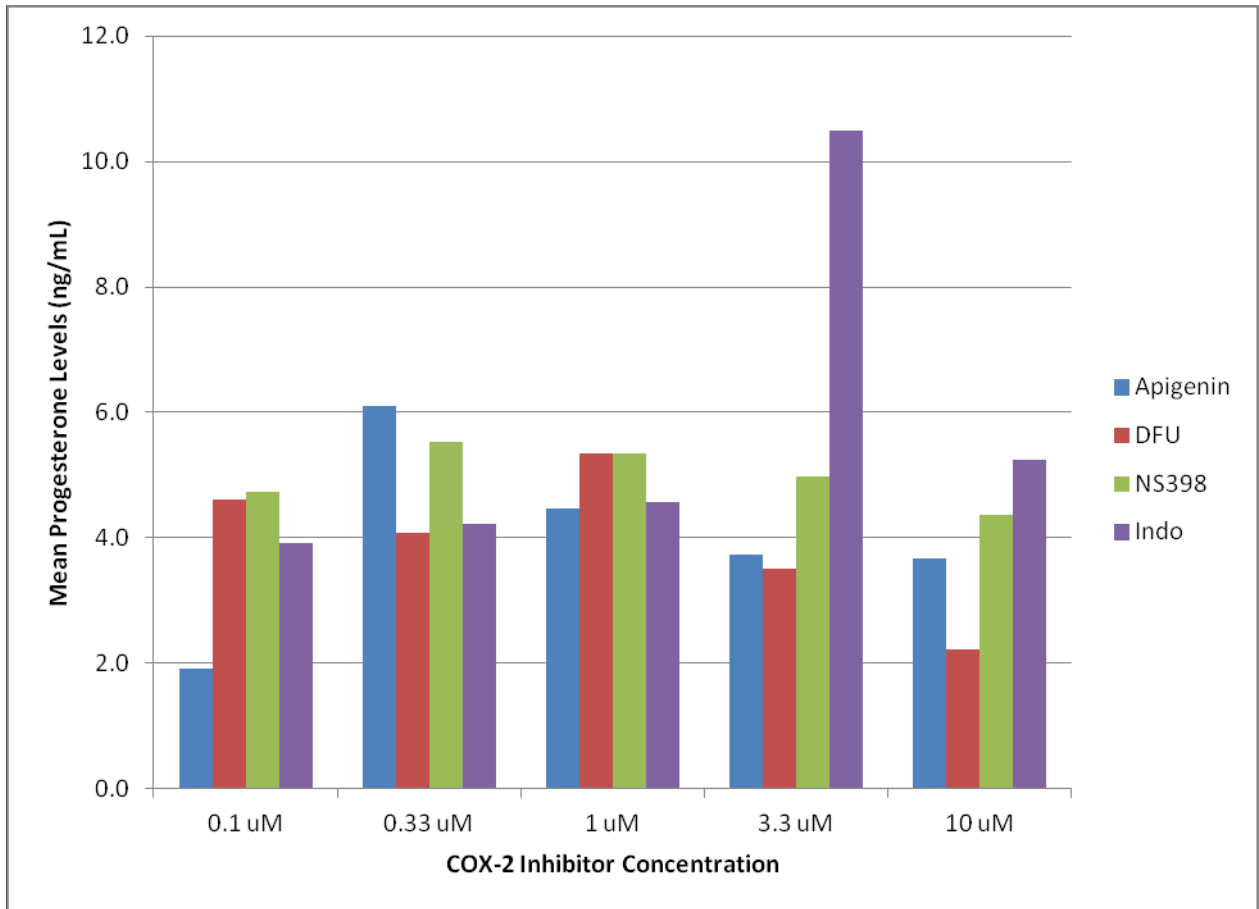


Figure 2A: Mean progesterone levels produced by MA-10 cells. Cells were treated with COX-2 inhibitor for 30 min, and then with a combination of inhibitor plus 0.1 ng/ml LH for 2 hours. COX-2 inhibitors used were Apigenin, DFU, NS398, and Indo. (n=2)

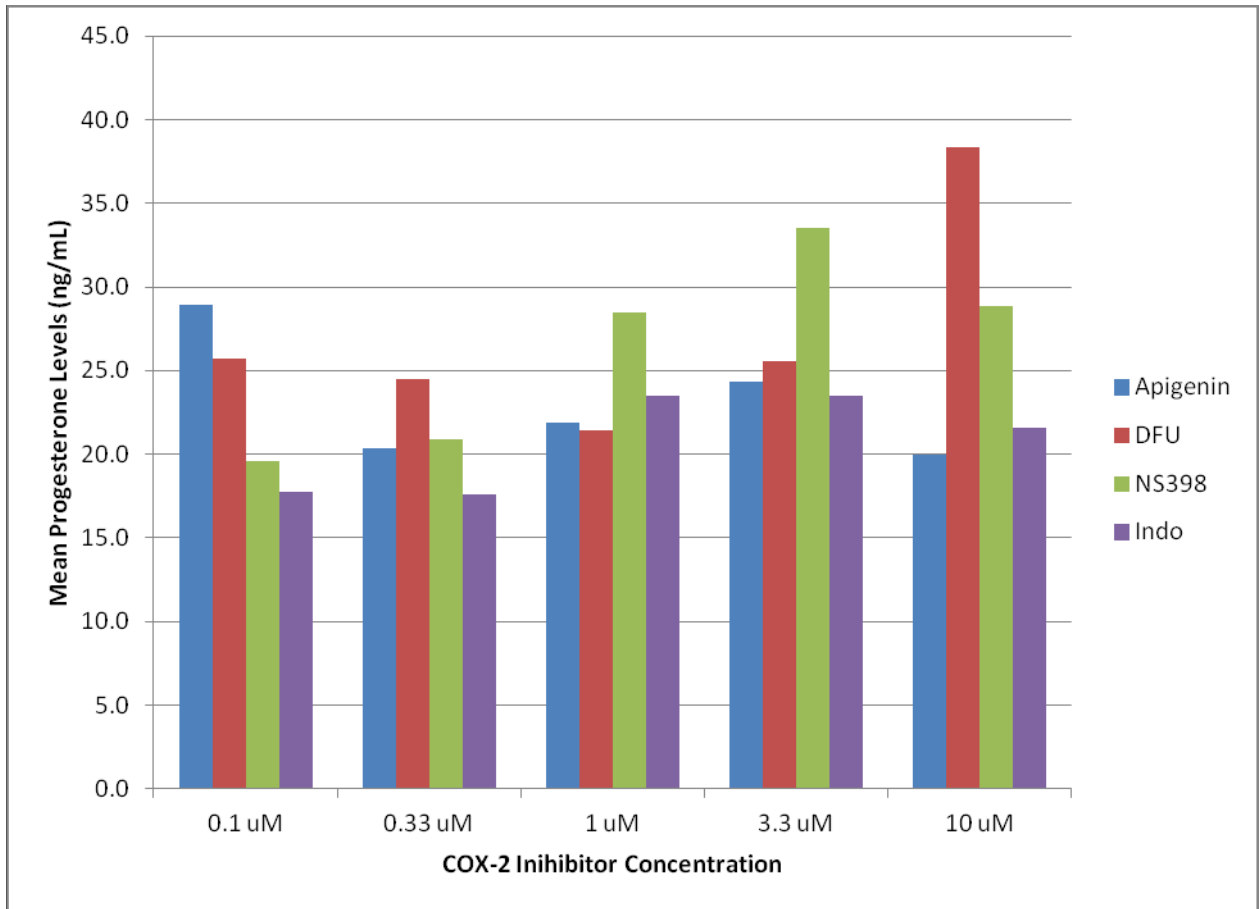


Figure 2B: Mean progesterone levels produced by MA-10 cells treated increasing concentrations of COX-2 inhibitors (30 min) and then with inhibitor plus LH for 2 hours. (n=2)

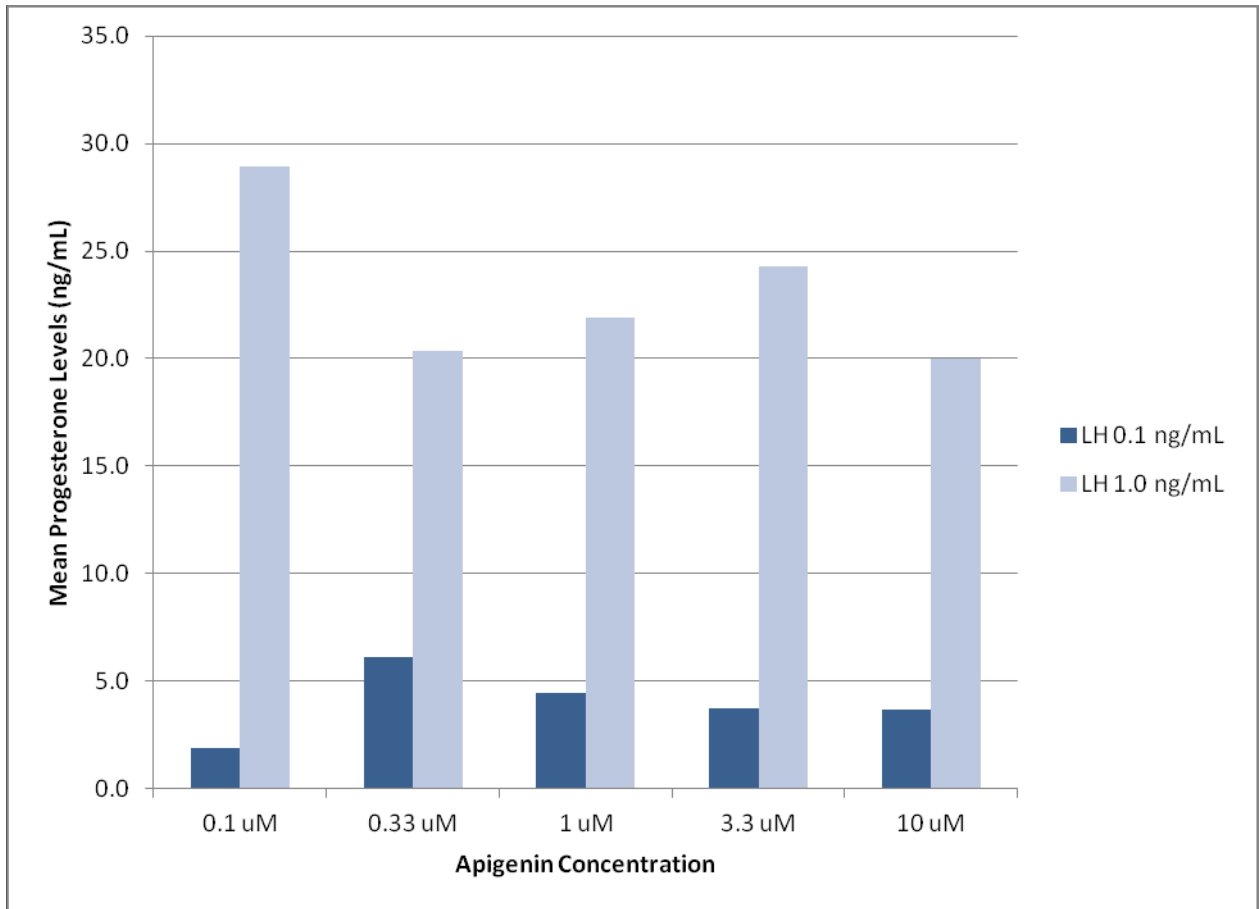


Figure 3A

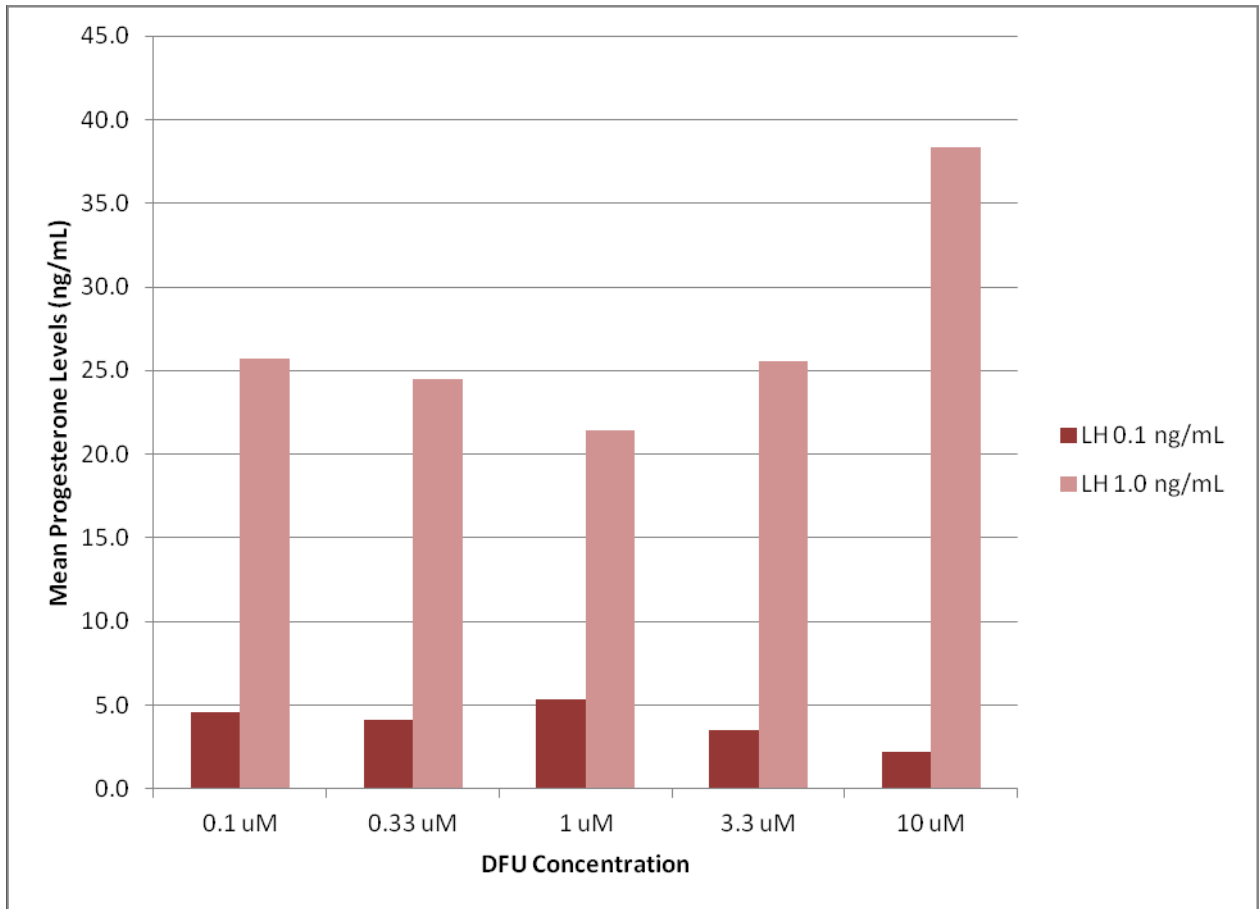


Figure 3B

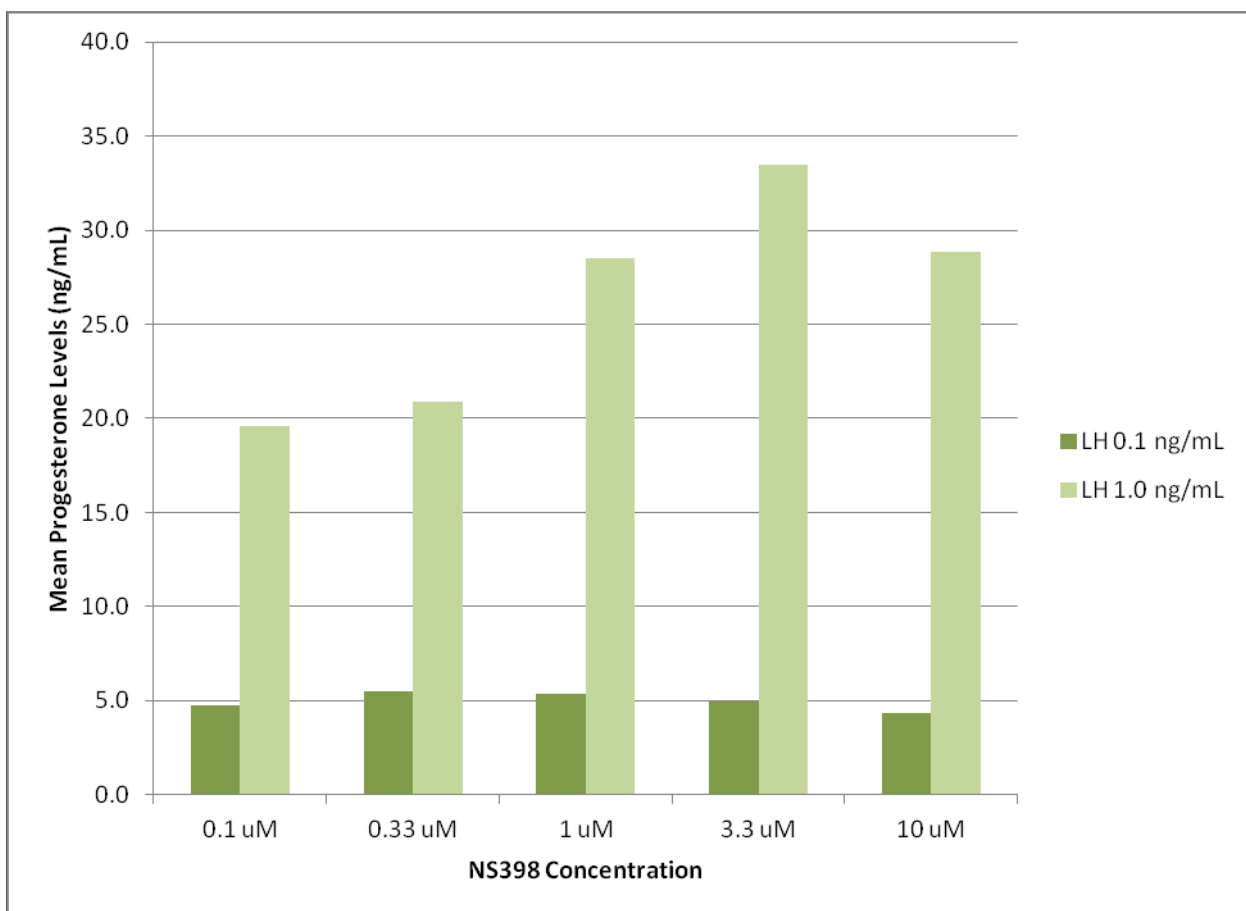


Figure 3C

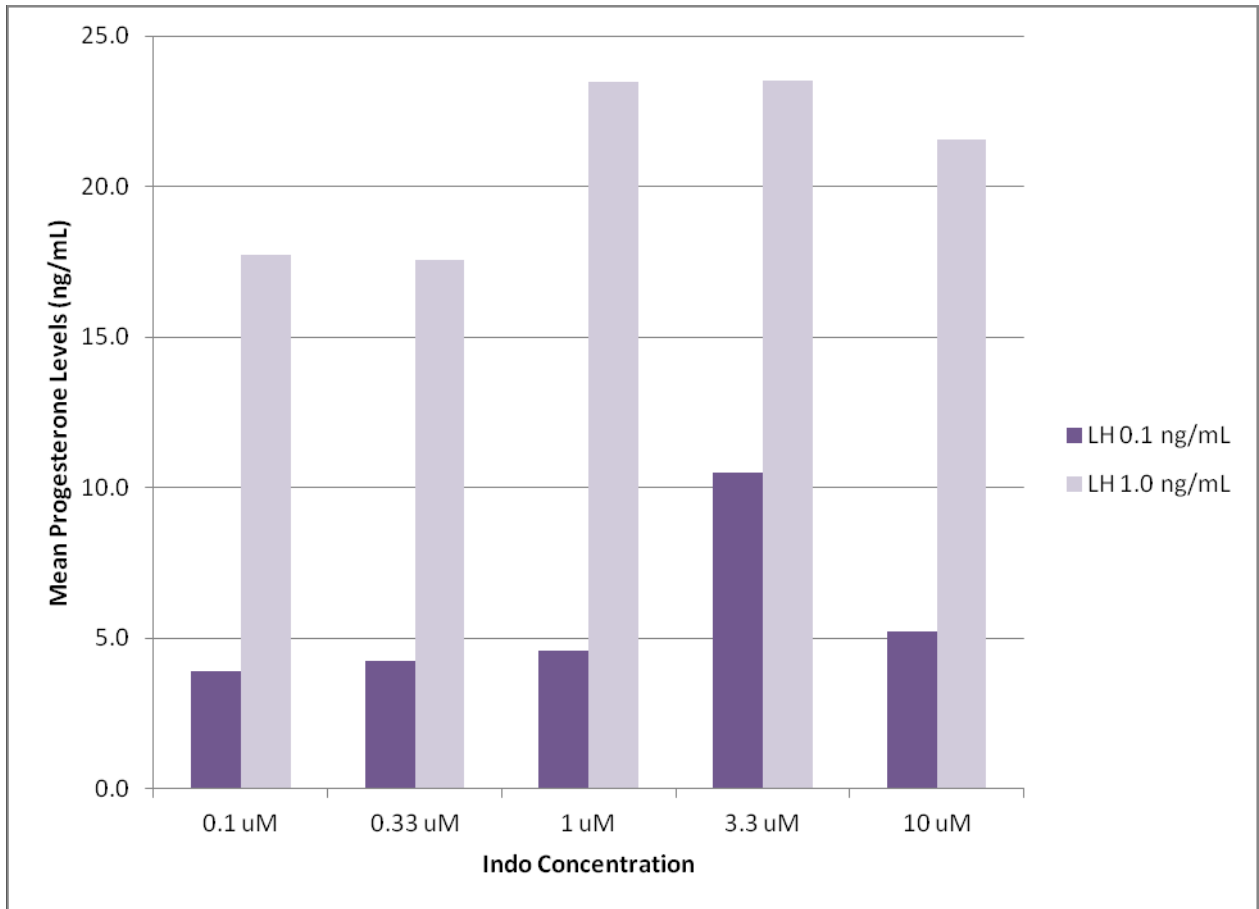


Figure 3D

Figure 3A-3D: Comparison of progesterone levels produced by MA-10 cells treated with individual COX inhibitors (30 min) and then inhibitor plus LH for 2 hours. (n=2)

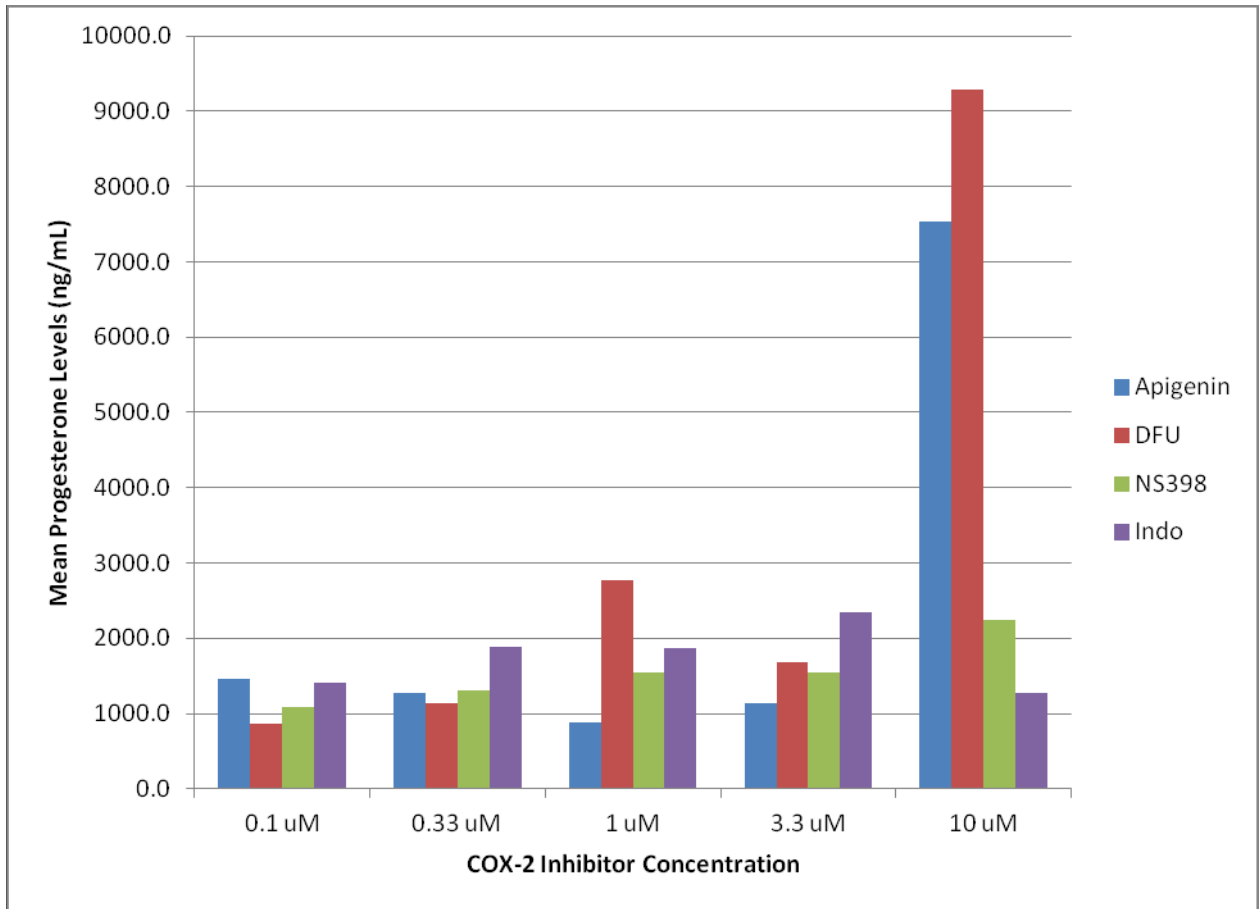


Figure 4A: Mean progesterone levels produced by MA-10 cells treated with COX-2 inhibitor (30 min) and then inhibitor plus dbcAMP (0.1 mM) for 2 hours. (n=2)

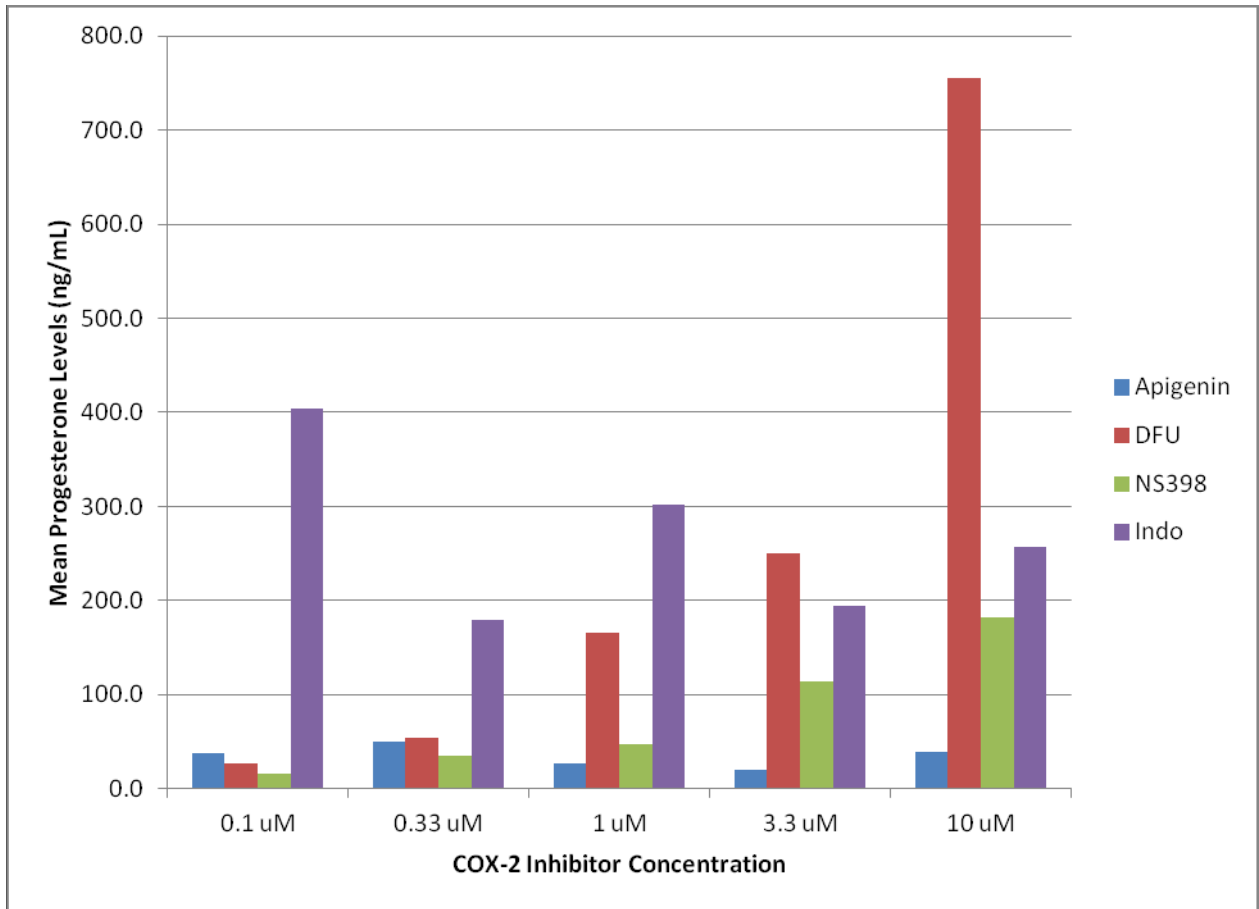


Figure 4B: Mean progesterone levels produced by MA-10 cells treated with COX-2 inhibitor (30 min) and then inhibitor plus dbcAMP (0.05 mM), for 2 hours. (n=2)

Possible COX-2 Inhibitor and TSPO Drug Ligand Treatment with LH or dbcAMP

TSPO Drug Ligand + LH or dbcAMP	COX-2 Inhibitor + LH or dbcAMP	Combination with LH Stimulation	Combination with dbcAMP Stimulation
Ro5 Alone FGIN Alone	DFU Alone Indo Alone	Ro5 + DFU Ro5 + Indo FGIN + DFU FGIN + Indo	Ro5 + DFU Ro5 + Indo FGIN + DFU FGIN + Indo
LH 0.1 ng/mL Treatment Regimen	LH 0.2 ng/mL Treatment Regimen	LH 0.5 ng/mL Treatment Regimen	dbcAMP 0.05 mM Treatment Regimen
Ro5 Concentration 20 μ M	Ro5 Concentration 20 μ M	Ro5 Concentration 20 μ M	Ro5 Concentration 20 μ M
FGIN Concentration 40 μ M	FGIN Concentration 40 μ M	FGIN Concentration 20 μ M	FGIN Concentration 20 μ M
DFU Concentration 10 μ M	DFU Concentration 10 μ M	DFU Concentration 10 μ M	DFU Concentration 10 μ M
Indo Concentration 10 μ M	Indo Concentration 5 μ M	Indo Concentration 2.5 μ M	Indo Concentration 5 μ M or 10 μ M

Figure 5

Figure 5: Combinations of treatments used when examining the effects of COX-2 inhibitor and TSPO drug ligand treatment on progesterone production. Final COX-2 inhibitor and TSPO drug ligand concentrations used are listed for each treatment regimen.

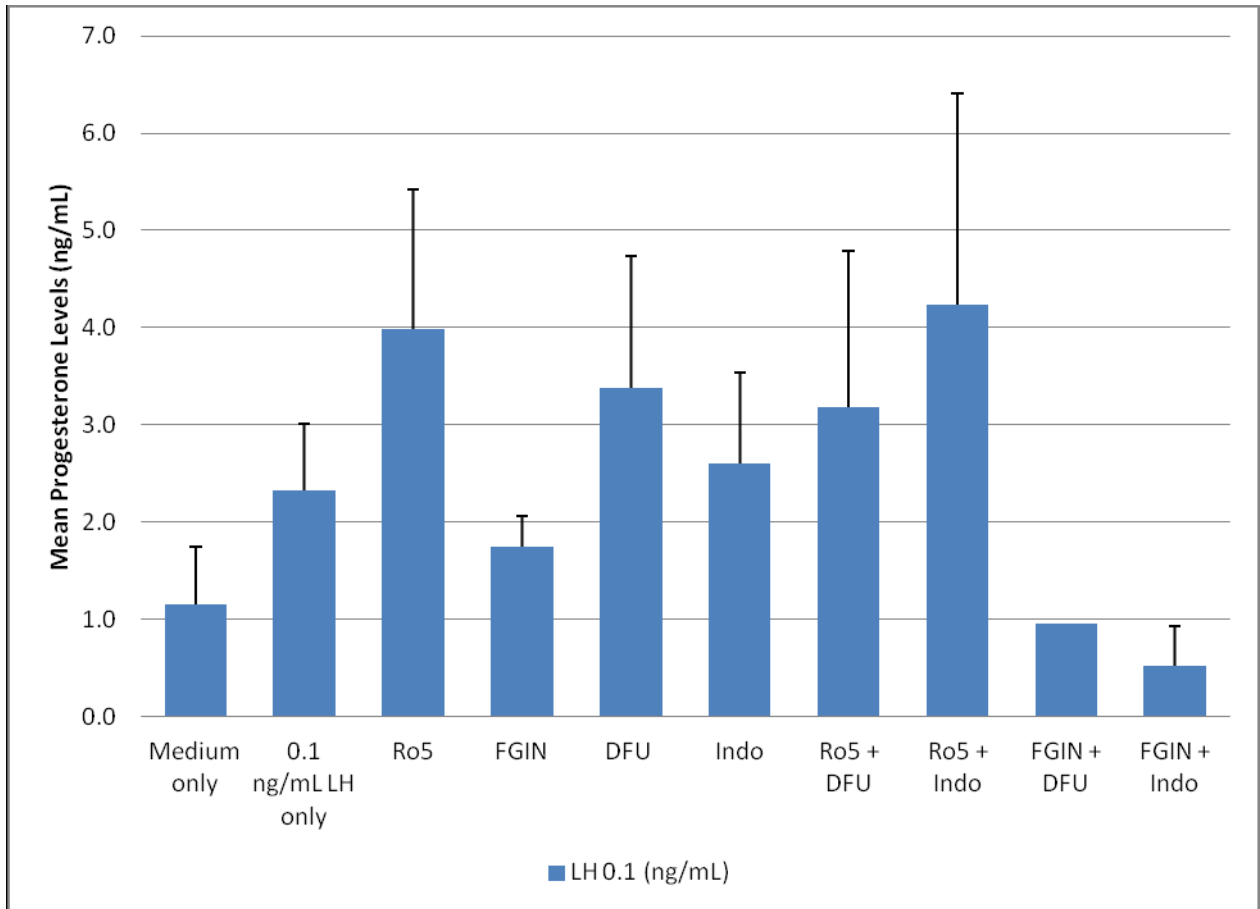


Figure 6A: Mean progesterone levels produced by MA-10 cells treated with TSPO drug ligands, COX-2 inhibitors, and LH at a concentration of 0.1 ng/mL. COX-2 inhibitors used were DFU and Indo. TSPO drug ligands used were Ro5 and FGIN. The data are expressed as a mean +SEM of at least 3 experiments. (n=3-6). Standard error bars were not expressed for FGIN + DFU treatment (n=2). No statistical difference was observed between treatments ($P>0.05$).

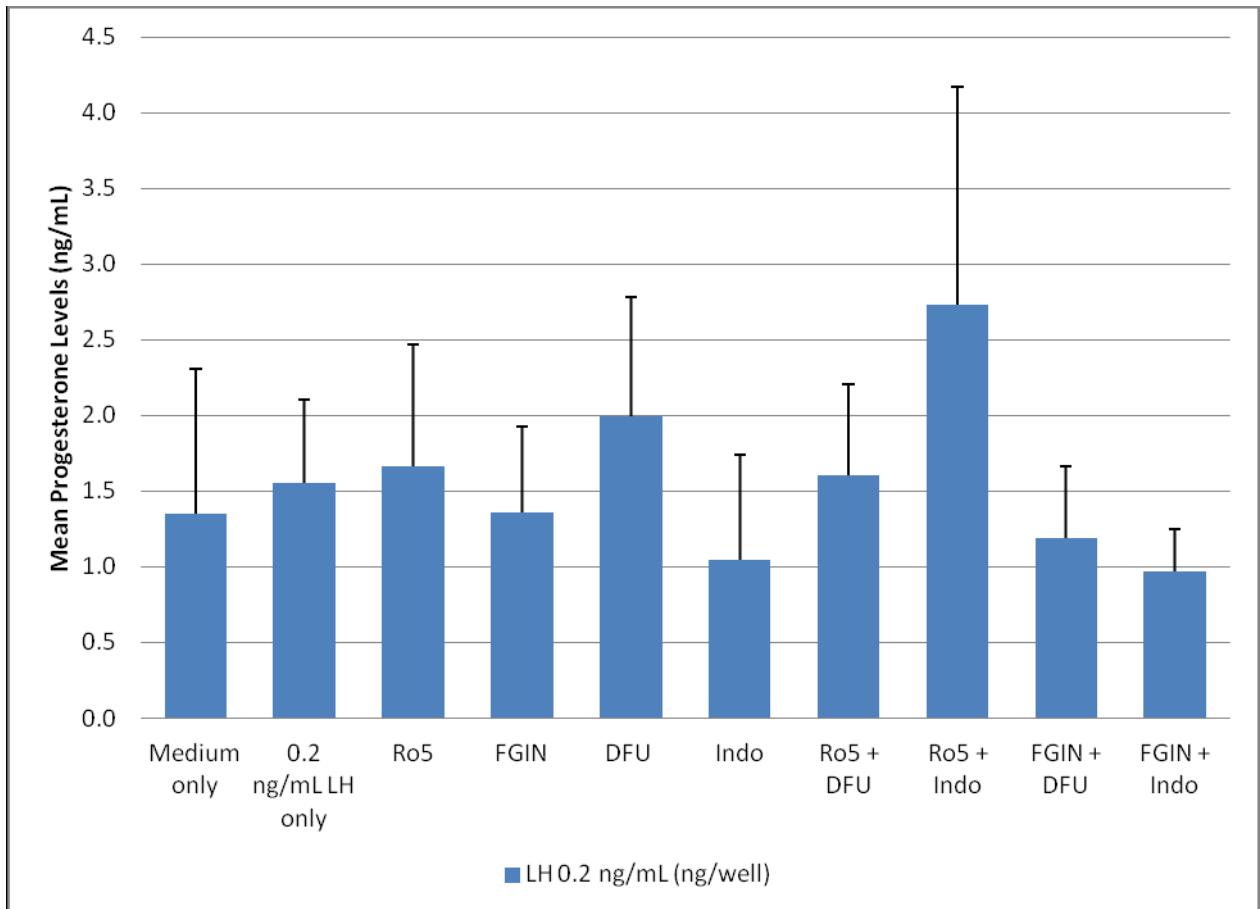


Figure 6B: Mean progesterone levels produced by MA-10 cells treated with TSPO drug ligands, COX-2 inhibitors, and LH at a concentration of 0.2 ng/mL. COX-2 inhibitors used were DFU and Indo. TSPO drug ligands used were Ro5 and FGIN. The data are expressed as a mean +SEM of at least 3 experiments. (n=4-9). No statistical difference was observed between treatments ($P>0.05$).

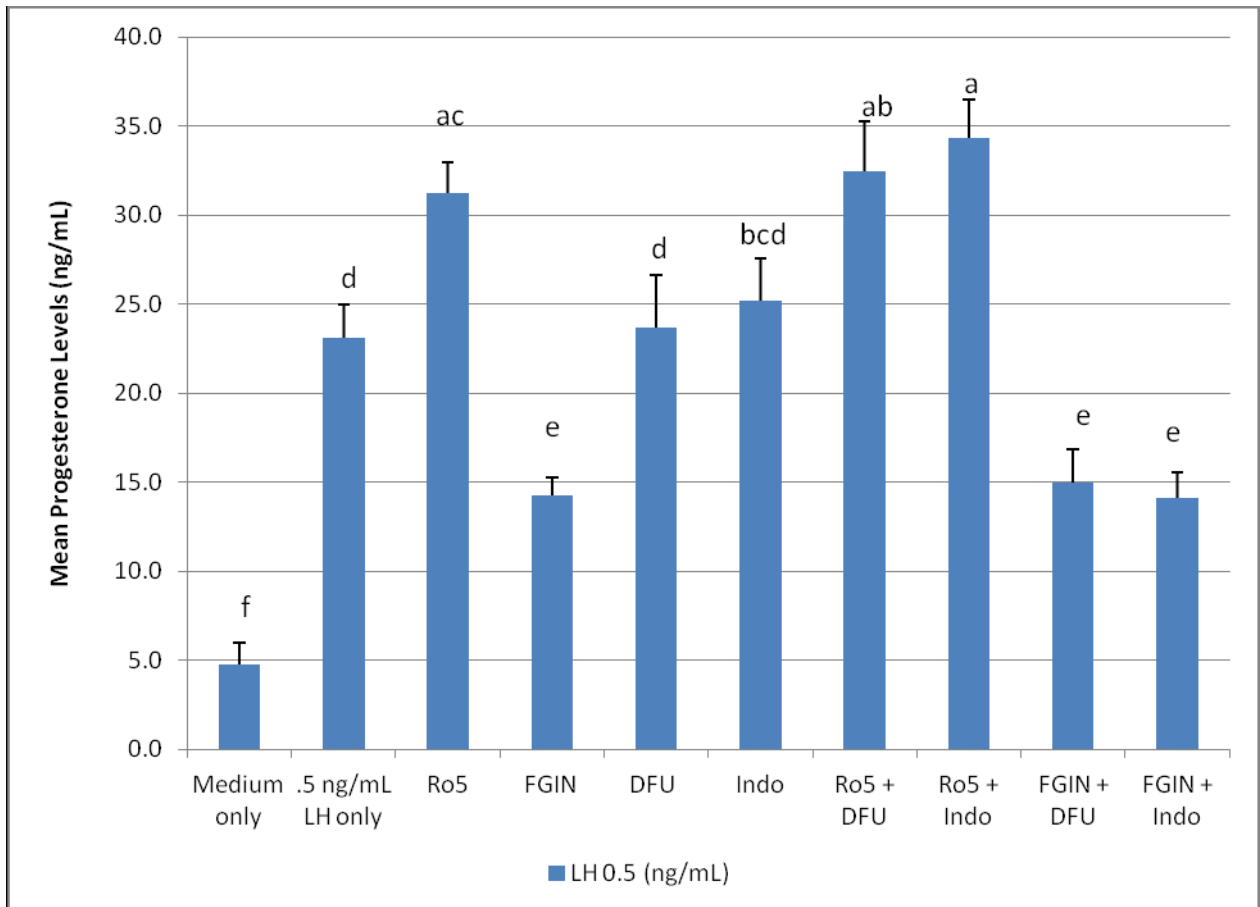


Figure 6C: Mean progesterone levels produced by MA-10 cells treated with TSPO drug ligands, COX-2 inhibitors, and LH at a concentration of 0.05 ng/mL. COX-2 inhibitors used were DFU and Indo. TSPO drug ligands used were Ro5 and FGIN. Results were measured with radioimmunoassay. The data are expressed as a mean +SEM of at least 3 experiments. (n=6-12). Statistical differences were observed between treatments ($P<0.05$), and groups that were not different are represented by the same letter

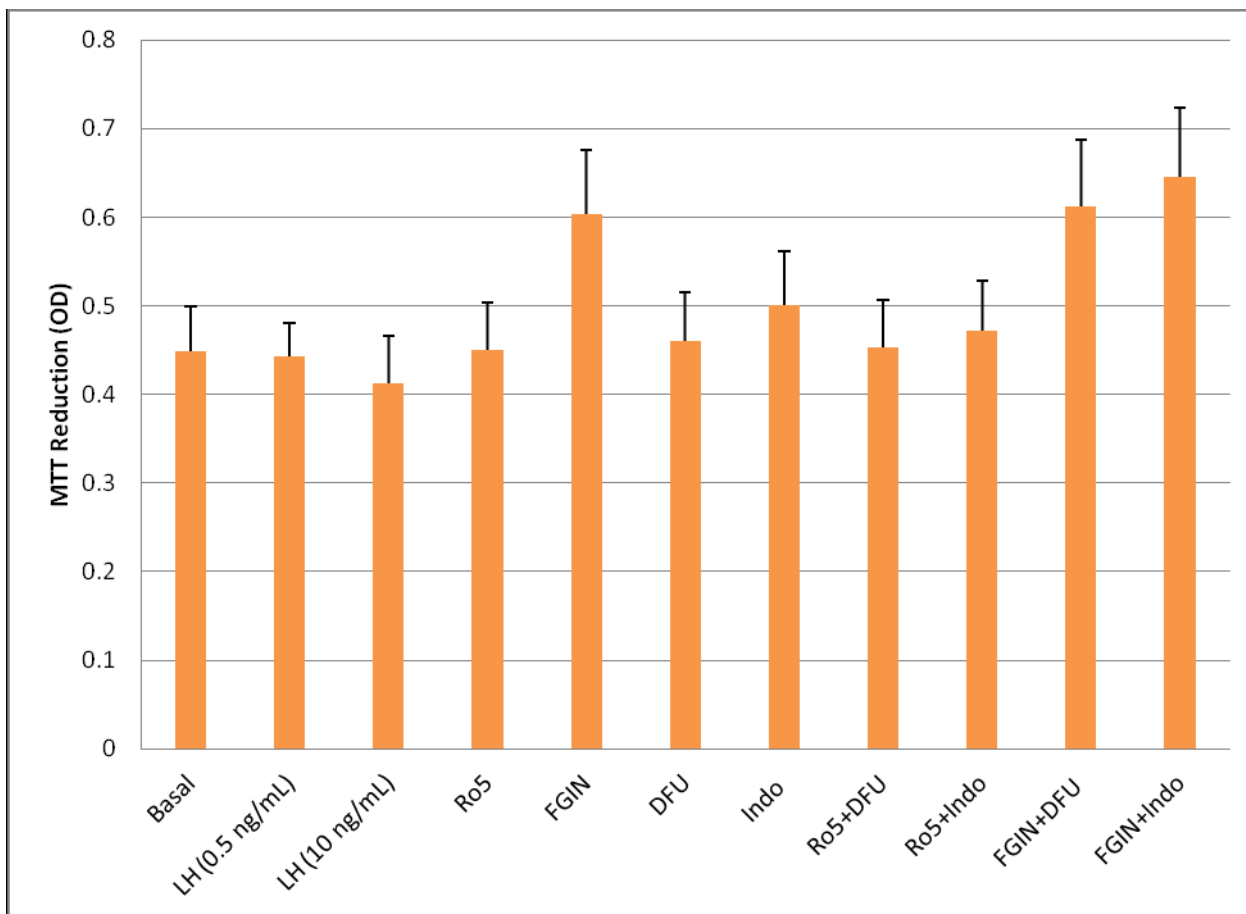


Figure 7: MTT Assay was performed on plates treated with COX-2 inhibitors and TSPO drug ligands in order to assess cell viability. COX-2 inhibitors used were DFU and Indo. TSPO drug ligands used were Fo5 and FGIN. After treatment, cells were provided with MTT for an additional hour. The reduction of the MTT dye by the viable cells was read on plate reader at 562 nm wavelength. The data are expressed as a mean +SEM of 3 experiments (n=12-24). No statistical difference was observed between treatments ($P>0.05$).

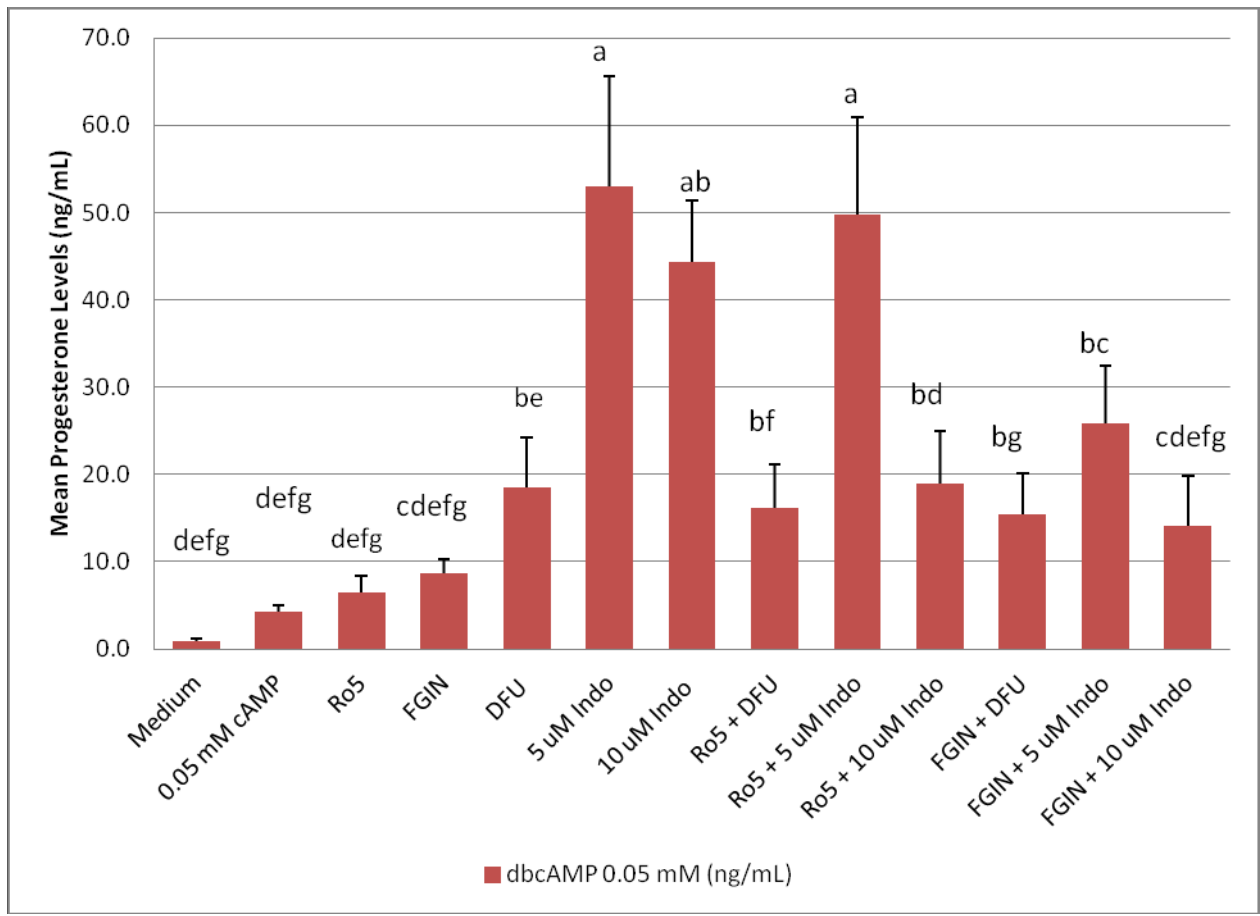


Figure 8: Mean progesterone levels produced by MA-10 cells treated with TSPO drug ligands, COX-2 inhibitors, and dbcAMP at a concentration of 0.05 mM. COX-2 inhibitors used were DFU and Indo. TSPO drug ligands used were Ro5 and FGIN. Results were measured with radioimmunoassay. The data are expressed as a mean +SEM of at least 3 experiments (n=3-24). Statistical differences were observed between treatments ($P < 0.05$), and groups that were not different are represented by the same letter.

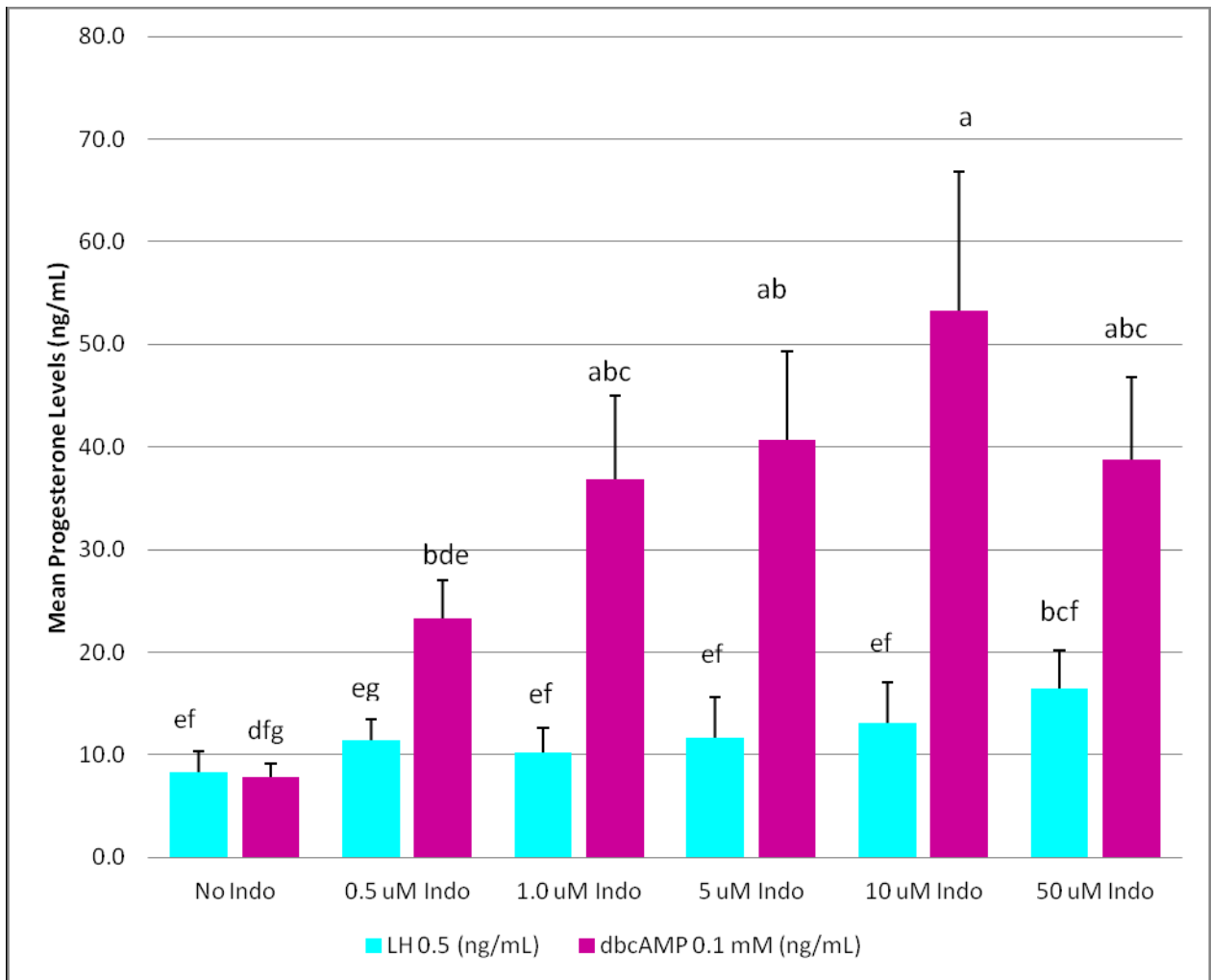


Figure 9: Mean progesterone levels in ng/mL produced by MA-10 cells treated with the COX-2 inhibitor Indo on a dose curve and LH at a concentration of 0.5 ng/mL or dbcAMP at a concentration of 0.1 mM. Indo concentrations ranged from 0.5 μ M to 50 μ M. Results were measured with radioimmunoassay. The data are expressed as a mean \pm SEM of 8 experiments. (n=8). Statistical differences were observed between treatments ($P < 0.05$) and groups that were not different are represented by the same letter.

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Birth October 2nd, 1988 in Tampa, Florida

Education

Expected June 2014 **Master of Science (Sc.M)**, Department of Biochemistry and Molecular Biology, Program in Reproductive and Cancer Biology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Research Based Thesis: Combined Effects of COX-2 Inhibitor and Translocator Protein Administration on Steroid Hormone Synthesis (Advisor: Dr. Barry Zirkin)

May 2011 **Bachelor of Science (B.S)** Neurobiological Sciences (Interdisciplinary Studies), University of Florida, Gainesville, FL

Senior Thesis: Comparison of Corticobulbar and Corticospinal Motor Deficiencies in a Rat Model of Stroke (Advisor: Dr. Jeffery Kleim)

Research Experience

June 2013- Present **Master's Student**, Lab of Dr. Barry Zirkin, Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

- Conducted an independent research project with a focus on steroidogenesis in various models of Leydig cell aging, with a particular emphasis on increased testosterone production through the action of translocator protein (TSPO)

June 2009- May 2011 **Research Assistant**, Lab of Dr. Jeffrey Kleim, Department of Neuroscience, McKnight Brain Institute, University of Florida, Gainesville, FL

- Conducted research on neural plasticity involved in cranial and limb motor function following an ischemic stroke of the middle cerebral artery
- Focused on rehabilitation in motor function following cortical ischemia in an animal model by using intracortical microstimulation to examine motor capabilities

Poster Presentation

Zuha Warraich, Nagheme J. Thomas, **Elizabeth Hernandez**, Rachel Allred, Jeffery A. Boychuk, and Jeffrey A. Kleim; Middle cerebral artery occlusion induces limb motor deficits and reduces forelimb motor maps but does not affect cranial motor function or oral motor maps. 40th Annual Meeting Society for Neuroscience, San Diego, CA, November 2010.

Technical Skills

- Small animal research (rats)
- Behavior Analysis techniques
- Neural tissue mounting
- Lesion reconstruction
- Motor map analysis
- Cell culture and maintenance
- Radioimmunoassay
- Protein analysis

Academic Achievement/Honors

August 2013- May 2014	Master's Tuition Scholarship Award
Fall 2010- Spring 2011	Dean's List
January 2009- May 2011	National Society of Collegiate Scholars
August 2007-May 2011	Member of the University of Florida Honors Program
August 2007- May 2011	Florida Academic Scholars Award
Spring 2007	National Merit Commended Scholar

Relevant Public Health Coursework

- Psychiatric Epidemiology
- Issues in Mental Health Research in Developing Countries
- The Public Health Approach to Psychopathology
- Introduction to the U.S. Healthcare System
- Fundamentals of Clinical Oncology for Public Health Practitioners
- Issues in the Reduction of Maternal and Neonatal Mortality in Low Income Countries

Clinical Experience

May 2011- June 2012

Clinical Technician, Florida Ophthalmic Institute, Gainesville, FL

- Responsible for obtaining full patient histories and beginning preliminary exams for all patients, ran tests ordered by the physicians, and assisted with office procedures
- Administrative duties included corresponding with other physicians and insurance companies, medical billing and record keeping procedures, and assisting the office manager
- Clinical research responsibilities included recruiting patients to participate in research trials, and working with companies to expand research projects taking place at the Florida Ophthalmic Institute

Leadership/Campus Involvement

Dec. 2009-Nov. 2011

House Manager, Executive Board Member, Delta Zeta Sorority, Pi Alpha Chapter, University of Florida, Gainesville FL

- Managed chapter operations and portion of \$80,500 budget for the 163 member chapter
- Managed house leases for 50 members
- Liaison between Delta Zeta National Housing Corps and chapter
- Negotiated a 50% reduction in rent price increases with National House Corps
- Served as Assistant New Member Education to the Vice President of New Member Education from January 2009-November 2009

April 2010- May 2011

Vice President, Lions Club International, Gainesville, FL

- Planned events designed to educate others about the blind community and assisted blind individuals during events such as Meet the Blind and MindSight at the Samuel P. Harn Museum of Art
- Acted as Co-Secretary during August 2009-April 2010

Sept. 2008- May 2009

Undersecretary to the Environmental Affairs Cabinet, University of Florida Student Government, Gainesville, FL

- Assisted in planning and participated in events geared towards making the University of Florida a more sustainable campus; promoted sustainable behavior amongst students

January 2008- May 2008
Gainesville

Arts in Medicine Volunteer, UF Health Shands Hospital,

- Put on improvised musical concerts for Bone Marrow Transplant Unit patients and their families; aided Artists in Residence with art, music, and dance activities around the hospital